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AN INVESTIGATION OF THE FREEZE TEXTURIZATION MECHANISM IN FOOD PROTEINS

by

(C)

Frisco Consolacion

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF **Doctor of Philosophy**

IN

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Department of Food Science

EDMONTON, ALBERTA

Fall 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **AN INVESTIGATION OF THE FREEZE TEXTURIZATION MECHANISM IN FOOD PROTEINS** submitted by **Frisco Consolacion** in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** in **Food Processing.**

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Supervisor

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Date..July 3/86

ABSTRACT

This study was conducted to elucidate the freeze texturization mechanism in poultry meat proteins. This objective was met to some extent by studying the effects of the alkali and acid treatments on fiber formation, effect of the freezing modes (non-directional and uni-directional) and freezing temperature (-25 and -60°C), and the effect of pH (5, 7 and 9); in the presence of calcium or urea, both on the structure and texture of the protein matrix. The interpretation of the results was based on the interrelationships of chemical composition, microstructure and texture of the protein system.

The SDS-PAGE of the fresh protein isolate showed the presence of high molecular weight protein subunits (18,000-248,000 daltons). Fiberization and extended cross-linking of these proteins, through the formation of secondary bonds, in the fresh protein isolate and freeze texturized product were revealed by using the scanning electron microscope. Non-directional freezing (at -25°C) of the protein slurry yielded textured product possessing low textural strength, which was further reduced at -60°C. Under alkaline environment or pH above the isoelectric point of the major meat protein components (myosin and actin) and in the presence of urea, the texturized products were characterized by gross alterations in the structure of the protein matrix, as viewed on the macrolevel. The loss of structure under alkaline conditions was restored upon addition of calcium

ions to the protein system. The effect of added calcium was manifested in the higher textural strength compared to the texturized samples without calcium and at alkaline pH. However, the additional strength provided through calcium binding was not sufficient to provide the textural strength of the protein slurries texturized at pH 5 and in the absence of this bivalent cation. Microscopic analysis revealed formation of numerous microcavities, flattened protein sheet structures (main and interconnecting structure) and, in some instances, disorganized sheet structures. These structural changes were in turn demonstrated in the significant decreases in the textural strengths of the final textured protein. The data obtained as a result of the various treatments of the protein slurry suggested the important participation of some chemical bonds, such as ionic, hydrogen and hydrophobic bonds, in protein texturization. It has been confirmed that protein slurry (pH 5) is the optimum substrate for the freeze texturization process, wherein the ability of the untextured or disorganized protein mass to rearrange into a new organized structure has been demonstrated.

The success in freeze texturizing alkali-treated poultry deboning residue proteins and the failure to texturize mechanically deboned poultry meat paste and the different whey protein preparations were speculated to be related to conformational differences among the test proteins.

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5

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1. INTRODUCTION

1.1 Texturized Protein Foods

In recent years substantial effort has been directed to the possibility of fabricating protein-rich foods from various sources in order to maximize utilization of the surplus, inexpensive proteins that would not otherwise be used for human foods. Many protein sources, such as animal and plant protein by-products and single cell proteins, have not been fully utilized for human food because of the failure to duplicate the mouth-feel and the fibrous character of meat texture. Most of the protein concentrates are in a paste or an amorphous powdered form and thus would require processing to provide the texture or fibrous quality appealing to consumers (Hoer, 1972). Although a variety of the conventional and non-conventional protein sources are available as raw materials for the production of textured food products, there are important requirements that have to be satisfied before they can be used for this process. Some of the basic structure requirements for a polymer to be fiber-forming include a high molecular weight (greater than 10,000 daltons), long linear chain length with a high degree of linear symmetry, a low ratio of bulky side chains, and high degree of polarity. These structural requirements are necessary for the development of orientation and crystallization among the molecular chains (Hartman, 1978).

Numerous workers have developed a wide variety of techniques, including fiber spinning, thermoplastic extrusion and freeze texturization, for obtaining the required fibrosity in the simulated meat from the original untextured protein slurry or solution. The fiber spinning method for proteins was patterned by Boyer (1954) after the processing of artificial textile fibers. This process provides uniform fibers, however, it requires expensive spinning equipment and produces fibers that are soft and fragile (Arima and Harada, 1971; Lange, 1974). One alternative method, thermoplastic extrusion, describes the production of a proteinaceous meat-like product by extruding the dough into an area of reduced pressure (Kim and Lugay, 1977). The sudden drop of pressure, and flash evaporation of moisture, cause the development of an expanded "open-cell" structure. This process yields pieces of analogue which, after rehydration, have a chewy texture and appearance similar to that of meat (Atkinson, 1970; Kinsella, 1978). Based on this process, Strommer (1975) used the term "texturizing" to describe the process of changing the discrete portions or particles of proteins into chunks having continuous phase protein and expanding or puffing the protein material to produce cellular structure. On the other hand, freeze texturization is actually an old process, used even in ancient Japan for the production of a freeze-texturized soy protein product known as "kori-tofu". This product is characterized by having a sponge-like texture, devoid of the desirable meat texture. However, the orientation of the frozen protein material can be controlled by the freeze-alignment process introduced by Lugay and Kim (1981). This method was the basis of a study conducted by Lawrence and Jelen (1982), involving the effects of pH, total solids and freezing rate on the texturization of alkali-extracted meat proteins. The results obtained by these authors suggested a positive role of ice crystal formation on texturization and cross-linkage between fibers of the main fiber structure that was favoured at higher pH values. Further investigation on the nature of chemical interactions during freeze concentration was recommended by these latter researchers.

1.2 Objectives of the Study

Although the formation of well-defined, well-ordered fibrous structures by the formation of ice crystals during unidirectional freeze texturization can be reproducibly achieved (Kim and Lugay, 1977; Kim and Lugay, 1978a,b; Middendorf *et al.*, 1975; Lawrence, 1981), little is known of the driving forces and mechanism underlying these structures. Protein texturization implies protein-protein interactions and the type and extent of protein-protein binding forces determines the structural as well as the physical and textural characteristics of the texturized protein product. Since the physical and textural properties are manifestations of the micro- and macrostructures and chemical composition of the product, it was considered appropriate as an overall objective of this study to determine the effects of the nature of proteins, type of the predominating chemical bonds and the factors promoting and disrupting these bonds, in order to elucidate the mechanism of protein texturization by the freeze alignment process. Specifically, this study was conducted with the following objectives:

1. To characterize the alkali-extracted, acid-precipitated protein from mechanically-deboned poultry meat residues.
2. To determine the effects of pH, freezing temperature and presence of calcium or urea on protein texturization.
3. To demonstrate the importance of the alkali and acid treatments on protein interactions and texturization.
4. To illustrate, by scanning electron microscopy, the events leading to texture formation during freeze texturization of the alkali-solubilized poultry meat proteins.
5. To compare the texturizing capabilities of meat proteins and cheese whey proteins.

2. REVIEW OF LITERATURE

2.1 Texture Formation from Proteinaceous Materials

It is well established that proteins are an essential component in human nutrition and that proteins in the form of meat are the most attractive of all foods to the average human taste (Gutcho, 1977). Animals are inefficient in converting the proteinaceous vegetable material into meat in terms of food input to food output (Gutcho, 1977; Plaskett, 1977; Kim and Lugay, 1978), which has led to the high and escalating cost of meat, making it prohibitive in various areas of the world. Coupled with the growing problem of shortage of protein for human consumption, there is a need to maximize the use of proteins recoverable from food industry by-products (Young and Lawrie, 1975a) and to improve the acceptability of vegetable proteins (Hoer, 1972; Lecluse, 1975). To this end, the food industry has expended a great amount of effort and money toward developing meat-like substitutes from highly nutritious, but generally less desirable food materials (Gutcho, 1972; Middendorf *et al.*, 1975).

Texturization of food proteins involves manipulation of the physicochemical properties of the proteins and transformation into a desirable food item (Kinsella, 1978). Kinsella stressed that alteration in the proteins is limited only to secondary and tertiary structures for restructuring proteins in food fabrication.

The term "extruded protein products" denotes those protein-rich items which have been modified in structure, shape, texture, flavor, and appearance to simulate conventional food items, especially meat products (Kinsella, 1978). Kazemzadeh *et al.* (1982) considered that a product has been "texturized" if it is able to withstand a specified testing process of hydration, retorting, grinding, and screening. Also, in the case of extrusion-texturized plant proteins, these products are considered to have been texturally and histologically restructured to give textural properties similar to those of muscle meat. Clearly, untexturized protein material contains protein in discrete and separate particles, and texturization is said to take place when the protein acquires a substantially continuous phase (Strommer and Beck, 1973).

Texture is the most important quality attribute of meat (Stanley, 1983), thus, this physical property is considered as the main determining factor in assessing the success of any protein texturization process. In fact, despite the controversial issues about cholesterol and saturated fat, and in the face of escalating meat prices, a major upsurge in the consumption of soy protein in the form of meat analogue has not yet occurred. This, according to Stanley (1983), implies the failure of soy products to significantly duplicate the texture of meat.

In general, textural properties of foods are perceived by our physiological senses as a direct result of interactions among the basic chemical components which give rise to the physical structure (Kazemzadeh *et al.*, 1982). DeMan (1976) defined texture as the way in which the structural components of a food are arranged in a micro- and macrostructure and the external manifestation of this structure.

All meats, including fish and poultry, have fibrous structures. The texture of the meat products is inherently dependent upon the fibrous nature of meat (Kim and Lugay, 1977). Likewise, the presence of fibrous structure is an important factor in fabricated meat-like products. Thus, in producing these meat-like products, e.g. meat analogs, much effort has been directed to creating fibrous structure similar to natural meat (Boyer, 1954; Lecluse, 1975; Kim and Lugay, 1977).

2.2 Physicochemical Aspects of Protein Texturization

The modification of proteins to produce fibrous or "fiber-like" textured products is a subject of unique importance and interest. Very little is known about the mechanisms that produce the texturized protein fibers or the forces that hold the polypeptide chains in a given structure. In general, the term "fiber" denotes any filament whose length is much greater than its diameter. Shen and Morr (1979) proposed some types of molecular arrangement during the fiber forming process (Figure 2.1). The random arrangement of the molecules results in amorphous filaments

Figure 2.1 Proposed molecular arrangements for drawn filaments: (A) random arrangement of globules, (B) random arrangement of random coils, (C) and (D) crystalline filaments, and (E) partially crystalline filaments (Shen and Morr, 1979). Note: The schematic diagram described in this figure has been removed because of the unavailability of copyright permission.

(A and B), whereas an ordered molecular arrangement forms crystalline fibers (C and D). A partial crystalline filament results from the mixture of crystalline and amorphous areas in the structure. Shen and Morr used the term fiber to designate only those filaments that have an appreciable degree of crystallinity along their filament axis. In protein texturization for fabricating meat-like products, Kinsella (1978) suggested that the term filaments is more accurate since most, though not all, of the products possess the structural characteristics of fibers.

The principal stages of protein fiber formation by the spinning process were defined by Lundgren (1949) as the unfolding of protein chains using denaturing agents such as heat or alkali, the orientation of these unfolded molecules by the action of intense shear achieved by extrusion through small orifices, the fixation of the structure formed by coagulation or precipitation and the stretching of the fibers (Culioli and Sale, 1981).

2.2.1 Factors favoring fiber formation

Large molecular weight is a requirement for fiber formation (Shen and Morr, 1979). For proteins this represents a minimum chain molecular weight of 7,000 daltons. The tensile strength of the fibers increases with increasing subunit chain length until a limiting plateau is reached at ca. 200 amino acid residues. Further, Shen and Morr stated that longer subunit chain length does not increase the tensile strength of the resulting fiber. Much longer subunit chains are detrimental to fiber formation (Huang and Rha, 1974). An earlier study by Peters (1963) concluded that an average molecular weight in the range of 10,000 to 50,000 daltons is required for fiber formation. Below 10,000 daltons, only weak fibers, if any, can be formed, while above 50,000 daltons, high viscosity and gelation inhibit proper alignment and polymerization of the subunits to form the fiber.

Lundgren (1949) enumerated the molecular criteria which were best for optimum fiber formation, by the fiber spinning technique. The molecules should be linear (1,000 Å) and of molecular weight around 20,000 to 30,000 daltons. The molecules should possess a high degree of linear symmetry and be devoid of bulky side-chain groups that would interfere with chain opposition, lessen intermolecular association and bonding, and weaken crystalline forces, thereby weakening the fiber. The component molecules should have a high content of polar amino groups to enhance intermolecular cohesive forces. The presence of cysteine residues, which engage in disulfide bond formation, also strengthens the fibers.

Differences in amino acid composition and their arrangement and sequence affect protein conformation. This, in turn, governs secondary and tertiary structures, which affect the ease and extent of intermolecular interactions that are important in the intermolecular binding of fibers.

For example, polypeptides with relatively high concentrations of serine, threonine, glutamine, and asparagine would have a marked propensity to form hydrogen bonds (Huang and Rha, 1974).

Hydrogen bonding is a very important source of cohesive force in fibrous polymers. The bonding may be intermolecular or, as in the helical polypeptides, intramolecular. The positive effects of hydrogen bonds on the fiber properties are reflected in high melting points, low solubilities, and high tenacities, but it is less clear whether the origins of these effects are separable in practice into dipolar and true bonding aspects (Huang and Rha, 1974; Goodman, 1963).

Fiber formation involves aligning of α-helices or extended random coils side by side and holding them there to form a crystalline filament (Peterlin, 1975; Goodman, 1963). Regularly spaced cohesive forces are needed to hold the aligned chains in place. If there are sufficient chain segments not held together by attractive forces or experiencing repulsive forces, rearrangement out of this aligned configuration to a more favorable nonaligned structure will occur. These cohesive forces include the electrostatic attraction of unlike charges, hydrogen bonding, apolar (hydrophobic) interactions, and cross-linking through disulfide or other groups. Intrachain or intramolecular cross-links will hold the chain or molecule in a folded fashion making it impossible to be fully extended. Thus, they tend to stabilize folded globular structures (Kauzmann, 1959).

The electrostatic forces between polar units are generally of considerably greater magnitude than the van der Waals forces and, in the majority of fibrous polymers, these latter forces are supplemented by cohesive effects induced by the permanent dipoles (Goodman, 1963).

In an aqueous environment, polar or hydrophilic groups are stabilized by interacting with water; whereas nonpolar or hydrophobic groups are more stable away from water. The hydrophobic bond is used to describe the gain in stability or the lowering of free energy on the transfer of nonpolar residues from an aqueous environment to the nonpolar interior of the molecule. It is widely recognized that hydrophobic bonding makes a major contribution to the stability of globular proteins (Kauzmann, 1959).

2.2.2 Modification of proteins for fiber formation

Atlas and Mark (1967) stated that, in order to increase the resistance of a polymeric system against softening, dissolution, and mechanical deformation, four mechanisms can be employed: crystallization; cross-linking; the use of suitable linear macromolecules; and incorporation of a reinforcing filler. In attempting to make fibers from non-fibrous proteins, several different treatments may be applied to achieve or support the reorganization of the polymeric molecules.

Heat encourages the peptide chain to assume a random-coiled form in a solution. Thus, chain unfolding generally takes place. On subsequent removal of heat interchain bonding occurs, imparting the crystalline structure (Vinogradov and Linnell, 1971).

Chiang and Sternberg (1974) reported that hydrophobic associations may be enhanced by treatments of fibers with heavy metal salts (e.g., mercury). Hydrophobic forces help stabilize polypeptides in the folded configuration. These forces become stronger with increasing temperature, reaching maxima around 60°C. They may be of some significance in the thermal stabilization of protein fibers. The marked capacity of urea to dissociate and solubilize protein from soy fibers demonstrated the importance of hydrogen bonding and hydrophobic associations in holding protein fibers together. Primary, covalent disulfide bonds are also significant in protein fibers as thiol reducing agents can dissociate proteins from spun fibers (Kelley and Pressey, 1966).

Mechanical orientation by stretching the fibers formed is believed to lead to a more uniform filament, organized in the direction of the fiber axis (Lundgren, 1949) or to increase the crystallinity in a polymer (Odian, 1970). This type of orientation often brings changes in mechanical or physical properties to the fiber. However, whether orientation leads to crystallization or not depends on the possibility of chains fitting into the lattice structure, as well as the characteristics of side chains, as noted earlier

(Mark, 1942). In the presence of bulky side groups, the crystalline formation is therefore inhibited by both steric interference and rotational restriction factors.

Unfolding of the protein chains is an essential first step in converting globular proteins into fibrous form. Sodium hydroxide, the most frequently employed alkaline agent, can solubilize most proteins and also unfold them, dispersing the chains (Wormell, 1954). Unfolding depends on such factors as the average length, kind and number of cross-links in the native protein.

To cleave the disulfide bonds, thioglycolate, sulfite and performic acid were used. Certain other agents have also been mentioned as favorable for denaturation, unfolding and solubilization of protein, such as urea, guanidine salts, formamide, sodium salicylate, detergents, and lithium iodide solution (Lundgren, 1949). Anfinsen (1961) remarked that using mercaptoethanol and related thiol reducing agents as agents for the reduction of disulfide bonds in proteins has these advantages: 1) complete disappearance of disulfide bonds with reasonable care and reaction time; 2) no side reactions; 3) no detectable covalent change other than that involved in the cleavage of disulfide bonds to sulfhydryl groups; and 4) reversible denaturation for protein like ribonuclease.

Disulfide bonds are the most stable covalent cross-link in protein (Benesch and Benesch, 1959). Lindley (1959) indicated that unfolding, i.e., breaking up the intramolecular disulfide bondings to yield sulfhydryl groups, followed by oxidation, produced the highest precentage of cross-linking in the wool fibers.

Flory (1953) stated that only three cross-links per molecule are necessary to tie a protein molecule into an insoluble three-dimensional gel network. Hydrocolloids or food binders which may provide adhesive or binding forces between the protein molecular chains are promising modification agents in facilitating the fiber formation in edible products.

2.3 Mechanism of Protein Texturization

The basic principle involved in protein texturization processes is to convert the native, non-fibrous protein into fibrous form, accomplished by a series of treatments that change the intramolecular stereochemistry and develop intermolecular arrangement of the polypeptide in the protein chains. The unfolding of the native globular protein followed by reorganizing into more aligned and cross-linked state imparts a higher degree of physical strength in the fibers (Huang and Rha, 1974). Hydrogen bonds, hydrophobic bonds, salt bridges, van der Waals forces and disulfide bonds are the forces that maintain the folded states of proteins (Wyn, 1973) that must be disrupted and regenerated during the protein structure transformation.

2.3.1 Fiber spinning

The overall mechanism that involves the transformation of native globular proteins into edible protein fibers is summarized schematically in Figure 2.2, which also describes the spinning process. In the first step, a strong alkali treatment (pH 9-12) dissolves and denatures or unfolds the native globular protein, resulting in a shearable solution of random coils. Unfolding depends on such factors as average chain length, and the kind and number of cross-links in the native protein (Huang and Rha, 1974). The alkaline condition also favors protein polymerization via formation of disulfide bonds (Fukushima, 1980; Kelley and Pressey, 1966). The sulfhydryl groups are the most reactive groups encountered in proteins and the disulfide bonds formed by them are the most stable covalent cross-link found in proteins (Benesch and Benesch, 1959). The intrachain or intermolecular cross-link will hold the chain or molecule in a folded fashion, making it impossible for it to be fully extended, hence stabilizing the folded globular structure. Shen and Morr (1979) suggested that the intramolecular disulfide cross-links, which can prevent complete denaturation to random coil, must be reduced. However, the unfolding of the tertiary or quaternary structure of the protein should be accomplished without excessive degradation of the molecular chain (Hartman, 1978). The alkali treatment of the protein (e.g., 90% protein in the case of soy protein isolate) produces a spinning dope containing approximately

Figure 2.2 Schematic representation of the fiber forming process. Transformation of a native globular protein into an aligned, stretched, and crosslinked protein fibers (Shen and Morr, 1979). Note: The schematic diagram described in this figure has been removed because of the unavailability of copyright permission.

20% solids. Then the dope is aged (with agitation) to allow the viscosity to increase (100 to 1000 poise) (Kinsella, 1978). This is due to the occurrence of protein-protein interactions via the sulfhydryl-disulfide interchange reactions, as in the case of soy proteins (Kelley and Pressey, 1966; Fukushima, 1980). The formation of protein network entraps some water molecules, and this results in an increase in the viscosity of the dope (Cheftel *et al.*, 1985).

The protein dope passing through the fine spinnerets (die of 1,500 holes, each of 0.003-0.01 inch dia.) causes the polypeptide chain to draw close together, favoring hydrogen and ionic bonding, and leading to the orientation of the molecules in the fluid state (Hartman, 1978; Kelley and Pressey, 1966). As the extruded protein emerges from the spinnerets, there is partial relaxation of the orientation of the polypeptide chains. Thus, in the third step, the filaments are set when the stream of protein dope emerging from the spinneret contacts the coagulating medium of weak acid bath; containing either acetic, lactic or phosphoric acid at a pH of 2-4 and calcium or magnesium ions. Immediate coagulation of the outer layer of protein occurs, accompanied by countercurrent diffusion of the alkaline dope solution from the coagulating fibers and infusion of the fibers with coagulating solution (Kinsella, 1978). Molecular orientation and crystallization take place in the coagulated state (Hartman, 1978; Shen and Morr, 1979).

After setting, further orientation of the molecules is obtained by stretching the freshly formed and relatively plastic filaments, and a stretching procedure (about 50-400% stretch) involving no more than leading the fibers away from the extrusion orifice at a rate of speed sufficiently great to prevent the fibers from being formed in a kinky condition, enhances the orientation, and consequently the strength (fourth step). If the filaments emerging from the dies are not stretched, they will tend to be weak, tender and inelastic. Texturized protein made from kinky or unstretched fibers will lack chewiness, whereas the product made from highly oriented fibers will have improved chewiness and a more meat-like texture (Boyer, 1954).

The filaments are annealed while under stretch by heating them to just below the fusion temperature to increase the degree of crystallinity. The length of heating step required to effect fusion is dependent upon several factors, such as the temperature used, the type of protein fiber and the size of the bundle of fibers (Boyer, 1954). Finally, the fiber is cured by cross-linking agents (e.g., formaldehyde, and hydrocolloids or food binders such as carageenan, alginates and fats) in order to stabilize the texture so that the product can withstand thermal processing or to make it insoluble in water.

Theoretically, most proteins should be capable of forming fibers when subjected to the spinning process. However, the ability of various proteins to unfold into long polypeptide chains at alkaline pH varies. Furthermore, the uncoiled polypeptides from some proteins in alkaline solution associated rapidly and become excessively viscous or form gels, thereby impairing the spinning operation (Kinsella, 1978).

2.3.2 Thermoplastic extrusion

Thermoplastic extrusion technology has been used to texturize many defatted vegetable protein ingredients, and produce many fibrous structures and meat-like textures (Rhee *et al.*, 1981). The process requires soy flour or grits to contain a minimum of 50% protein with a nitrogen solubility index of 50 to 70, a maximum of 30% insoluble carbohydrates, and less than 1% fat (Smith, 1975). The basic information concerning the chemical and physical changes occurring during the extrusion or texturization in the raw material is very minimal. Various mechanisms have been proposed to explain the fiber formation and finally the characteristic meat-like structure.

In the thermoplastic extrusion process, a protein-containing raw material is mixed with water to form a "dough" with 10-60% moisture and is then forced, by means of a compression screw operating within a heated barrel from 200 to 400°F and at least 1000 p.s.i. through an orifice. While inside the extruder barrel, the mixture is worked and heated, causing the protein molecules to denature and form new cross-linkages which result in a fibrous texture. In the case of defatted soy flour, hydration of the globular proteins (glycinins) in the aleuron granules occurs inside the extruder barrel. At this point, the protein molecules unravelled, followed by stretching due to shearing action of the rotating screw flites. The proteins become aligned in sheaths, then are compressed further and laminated longitudinally and are denatured at high temperature when passing through the die. At the instant of emergence from the die, pressure drops, moisture vaporizes and flashes off, and air-space vacuoles are produced within the laminated extrudate. The cooling, accompanied by vaporization, allows rapid thermosetting or solidification of the stretched protein fibers. A porous structure, with parallel arrays of lamellae of protein fibers, results (Rhee *et al*., 1981; Kinsella, 1978; Plaskett, 1977; Shen and Morr, 1979).

2.3.3 Freeze texturization

In freeze texturization the protein solution or gel is frozen such that ice crystal formation concentrates the protein into the unfrozen liquid phase (Figure 2.3). This process brings the protein molecules close together allowing protein polymerization to take place via the disulfide, hydrogen, ionic, and hydrophobic bond formation. In the manufacture of kori-tofu, unfolding of the globular proteins is accomplished by heating a 5-10% protein solution (pH 7.0) to 100°C for 1 minute (Hashizume, 1978). This process exposes the masked sulfhydryl groups needed for disulfide

Figure 2.3 Insolubilization of soybean protein during frozen storage. Transformation of protein solution or gel into a freeze texturized soy protein curd showing sponge-like texture (Fukushima, 1980; Hashizume, 1978). Note: The schematic diagram described in this figure has been removed because of the unavailability of copyright permission.

bond formation. This stable covalent bond plays an important role in the texture of the product (Saio, 1969, 1971). The heat denatured soy protein is cooled immediately to less than 20°C and then $CaCl_2$ is added at the rate of 2.5×10^{-4} moles/g of protein. After freezing at -20°C the frozen gel is stored at -1 to -3°C for about three weeks to allow further polymerization of the proteins (Hashizume, 1978; Fukushima, 1980).

The proposed mechanism of the unfolding of the native protein molecules and their intermolecular polymerization is schematically presented in Figure 2.4. The existence of large numbers of disulfide bonds in each molecule suggests that interchain disulfide polymerization of the heated soymilk protein occurs through an interchange reaction between the sulfhydryl and disulfide groups. One or two sulfhydryl groups in each molecule could react readily with any of the several accessible disulfide bonds of another molecule and, consequently, the interchange reaction between the sulfhydryl and disulfide groups occurs to form new molecular bonds. By this reaction a new free sulfhydryl groups appears which can take part in another intermolecular reaction with disulfide bond and new sulfhydryl groups. Through this interchange mechanism, the intermolecular disulfide bonds can link at multiple sites on each molecule, resulting in a three-dimensional polymerization and insolubilization of the protein molecules. There were two proposed mechanisms for the polymerization of soy proteins, the first

Figure 2.4 Schematic diagram of unfolding of native protein molecules and their intermolecular polymerization: (1) unfolding of a polypeptide chain in a native molecule, (2) intermolecular polymerization through -SH/-S-S- interchange reaction, and (3) intermolecular polymerization through hydrophobic interaction (Fukushima, 1977).

Note: The schematic diagram described in this figure has been removed because of the unavailability of copyright permission.

being discussed above, while the second involves formation of disulfide bond by oxidation between the two free SH-groups located on different protein molecules. Based on the studies conducted by Fukushima (1980), the presence of only one or two free SH-groups per molecule of soymilk protein rules out disfulide polymerization via the second mechanism. Because the probability that one or two SH-groups will react intermolecularly is small and even though two SH-groups might react intermolecularly, the molecules will polymerize only to dimers when each molecule has one SH-group, and one-dimensional polymers only if all the molecules contain two SH-groups. Therefore, large amounts of insolubilized protein through this mechanism cannot be expected (Fukushima, 1980).

The sulfhydryl-disulfide interchange reaction has been suggested to occur also in the unfrozen liquid portion during the freeze-texturization of soy protein (Hashizume *et al.*, 1974; Fukushima, 1980). The possibility of protein polymerization through hydrophobic interaction was also considered (Figure 2.4). Moreso, hydrogen bond formation should be enhanced at this low temperature in view of the negative enthalpy that characterizes the process (Taborsky, 1979).

In practice, the soy protein curd or solution is frozen in such a way that heat removal takes place in all directions, resulting in a spongy product (Figure 2.3). In contrast, a paralled-oriented protein mass which is retained after freeze drying can be obtained following the freeze-alignment process introduced by Lugay and Kim (1981) (Figure 2.5). The parallel orientation of the frozen protein mass can be obtained by cooling only one surface, which will cause the ice crystals to grow aligned and perpendicular to that surface. The ice crystals, which grow as "spears" in the proteinaceous material, exclude other substances, and water molecules from the bulk of the system migrate and fit on the ice crystal lattices (Lawrence *et al.*, 1986). This process concentrates both the protein and other solutes in the spaces between the ice crystals, forming distinctly aligned parallel zones, which remain after thawing. Owing to the fragility of the protein structure, setting by heat, chemicals or dehydration has been suggested (Lawrence *et al.*, 1986; Lugay and Kim, 1981).

Lillford (1985) reviewed the various methods of freeze-texturization of proteins. He stated that all the existing processes involved the following critical steps: separation of phases on freezing (ice and concentrated solute or suspension), orientation of ice crystals and hence passive orientation of the concentrated phases if fibrous final structure is to be achieved, and fixation of the structure by formation of new chemical bonds. From the various patents (Table 2.1) describing the texturing of both animal and vegetable protein slurries and mixtures of both, Lillford presented some technical details which are of considerable importance. He stated that slurries, not solutions, of

Table 2.1 List of selected patents covering some aspects of the freeze-texturization process.

1. Okimura, G.K. and Wilkinson, J.E. U.S. Patent #3,490,914 (1970). Preparations of Vegetable Protein Containing Food Products.
2. Boyer, R.A. and Middendorf, J.E. U.S. Patent #3,870,808 (1975). Method of Producing a Meat Stimulating Textured Food Product.
3. Middendorf, J.E., Waggle, D.H. and Cornell, A. U.S. Patent #3,920,853 (1975). Protein Food Product.
4. Livingston, R.M., Matthews, A.D. and Hall, D.N. British Patent #1,544,906 (1976). Textured Protein Food Product Preparation from Protein Solution.
5. Kim, M.K. and Lugay, J.C. U.S. Patent #4,001,459 (1977). Fibrous Protein Materials.
6. Kim, M.K. and Lugay, J.C. British Patent #1,537,173 (1978). Textured Fibrous Protein Product.
7. Kim, M.K. and Lugay, J.C. British Patent #1,537,736 (1979). Fibrous Protein Materials.
8. Noguchi, A., Kimura, S. and Umeda, K. U.S. Patent #4,136,210 (1979). Process for the Production of Textural Protein Food Material from Krills.

From Lawrence *et al*. (1986).

Figure 2.5 Schematic diagram showing parallel ice crystal formation in freezing protein slurry. Concentrated protein zones between the ice crystals are shown (Lawrence et al., 1986). Note: The schematic diagram described in this figure has been removed because of the unavailability of copyright permission.

proteins are retextured, containing between 15-30% (w/w) solids. Proteins at their isoelectric pH are recommended, normally in pH ranges of 4.0-6.0, since solubilization of the proteins occurs outside this range. Salt concentrations below 3% (w/w) are also suggested in order to optimize the product texture. Solubilization of the native globular proteins of plant origin as well as some animal proteins is also considered at higher salt concentrations. Further, Lillford stated that the freezing rate is not considered critical. However, this parameter can be controlled so that fine or coarse fibrous textures can be obtained. At this point, the work conducted by Lawrence (1981) proved indeed that freezing rate affects the texture of the freeze-texturized protein slurry. During slow cooling the rate of ice crystal growth is relatively high compared to the rate of nucleation, thus fewer but larger ice crystals will form. This will result in the formation of relatively thick protein sheets when freeze texturing protein slurry. On the other hand, rapid cooling produces numerous but small ice crystals, consequently, producing thin protein sheet structures.

2.4 Proteins

Proteins are a class of naturally occurring compounds of high molecular weight, designated also as biopolymers. They are extremely widespread in nature, being one of the essential constituents of the tissues of plants and animals since they are fundamental in all aspects of cell structure and function (Lehninger, 1978; Peters, 1963). Proteins consist of repeating units of amino acids, linked together by peptide bonds (-CO-NH-), which, upon acid hydrolysis, yield primarily the α-amino acid types (Lehninger, 1978; Huang and Rha, 1974). There are about twenty frequently occurring amino acids: glycine, valine, leucine, isoleucine, phenylalanine, proline, serine, threonine, cysteine, methionine, tryptophan, tyrosine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, arginine and histidine.

Proteins in their native states can be classified according to conformation (i.e., the characteristic three-dimensional state) of the polypeptide molecules, such as fibrous and globular proteins (Lehninger, 1978). Fibrous protein has its polypeptide chains more or less extended and coiled into a long helix. On the other hand, globular protein chains are more tightly coiled and packed into a spherical or elliptical shape (Huang and Rha, 1974).

There are specific terms commonly used to refer to different levels of protein structure. Primary structure refers to the covalent backbone of the polypeptide chain and the sequence of its amino acid residues. Secondary structure refers to the arrangements of polypeptide chains along one dimension, maintained by hydrogen bonding. Tertiary structure indicates how the polypeptide chain is bent or folded in three dimensions through side chain interaction and cross-linking of disulfide bonds. When the protein contains more than a single polypeptide chain, the organization resulting from the non-covalent interaction of macromolecule subunits is called a quaternary structure (Huang and Rha, 1974; Lehninger, 1978).

2.4.1 Protein denaturation

Protein behaves differently under various environmental conditions, under which its conformation can be defined (Lapanje, 1978; Kauzmann, 1959). The profound changes in protein conformation as well as other specific properties are collectively referred to as denaturation (Anglemier and Montgomery, 1976). Kauzmann (1959) defined denaturation as a process (or sequence of processes) in which the spatial arrangement of polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement. Specifically, denaturation is any modification of secondary, tertiary or quaternary structure of the protein molecule, excluding breakage of covalent bonds (Anglemier and Montgomery, 1976). It is a process by which hydrogen bonds, hydrophobic interactions, and salt linkages are broken and the protein is unfolded (Joly, 1965). Protein denaturation is a physicochemical process, which can be reversible or irreversible (Lapanje, 1978). This process is considered to be reversible if, on restoring reversibly the original conditions, the native conformation, along with other properties, is regained.

There are various means of denaturing proteins, for example, by heat, pH, urea, organic solvents, inorganic salts and detergents. Since excellent reviews of the modes and types of protein denaturation are available (Lapanje, 1978; Kauzmann, 1959; Fennema, 1977) only brief discussion will be presented here.

Thermal denaturation is brought about by increasing the temperature (Lapanje, 1978). The coagulation of the proteins at high temperatures is due to the polymerization reaction based on unaltered native molecules or at least molecules considering no change in secondary structure (Jaenicke, 1971). In proteins with disulfide bonds, sulfhydryl-disulfide interchange reaction resulting in cross-links may also take place (Tanford, 1968; Kelley and Pressey, 1966; Fukushima, 1980).

Protein denaturation, that is, partial or complete unfolding, is favoured both at low and high pH far away from the isoelectric point (Lapanje, 1978). This is attributed to the electrostatic repulsive forces between like charges (Anglemier and Montgomery, 1976). Under alkaline conditions, intermolecular disulfide bonds are readily formed, resulting in aggregation, gelation or precipitation (Lapanje, 1978; Hashizume *et al.*, 1974; Fukushima, 1980).

A globular protein could be unfolded by using urea due to the rupture of the hydrophobic and hydrogen bonds in the protein chain (Whitaker, 1977). The extent of unfolding depends not only on high concentration of urea (6-8M) but also on temperature, pH and the ionic strength of the aqueous protein solution (Anglemier and Montgomery, 1976; Lapanje, 1978). On the other hand, Finer *et al.* (1972) interpreted the action of urea on proteins in aqueous solutions in terms of its action on water structure, which in turn changes the nature of polymer-water interactions.

The action of organic solvents (e.g., alcohol) involves a hydrophobic bonding mechanism (Lapanje, 1978). This is increased by increased alkyl residue content in the protein molecule. Organic solvents possess both hydrophilic and hydrophobic regions, thus can penetrate the hydrophobic region of the protein molecule (Sosulski, 1977).

Synthetic detergents, both ionic as well as nonionic, are the most effective denaturing agents (Anglemier and Montgomery, 1976; Lapanje, 1978). These compounds have the ability to form a chemical bridge between hydrophobic and hydrophilic environments, thus disrupting or decreasing the hydrophobic forces needed to maintain the native protein structure (Anglemier and Montgomery, 1976). However, in contrast to denaturation by organic solvents, the conformation resulting from treatments of detergents is still ordered and not randomly coiled polypeptide chain (Lapanje, 1978).

Protein binds calcium, magnesium and many other multivalent ions; calcium binding is crucial to the stabilization of the casein micelle (Regenstein and Regenstein, 1984). Calcium bridging between negatively charged amino acid residues in soy protein is also responsible for the formation of protein coagulate, upon addition of calcium chloride in heated soymilk at pH 7 (Hashizume *et al.*, 1974). The large salting out (minimum protein solubility) effect of calcium chloride on nonpolar compounds was demonstrated by Nandi and Robinson (1972).

2.4.2 Protein sources

A variety of proteinaceous materials can be used to produce fabricated foods: vegetable proteins, such as soy, cottonseed, peanut, sunflower seed, rapeseed sesame seed; animal protein concentrates, such as egg albumen and casein; and microbial protein, from sources such as brewer's yeasts or torula yeasts (Boyer, 1956; Lange, 1974; Hoer, 1972; Satterlee, 1981). Proteins from meat industry waste, e.g. lung and stomach (Young and Lawrie, 1975a), blood plasma (Swingler and Lawrie, 1977), poultry deboning residues (Lawrence and Jelen, 1982a) and beef bones (Jelen *et al.*, 1979), are increasingly utilized to prepare simulated foods. Further, whey, a major by-product in cheese manufacturing, is also being considered as a potential protein source (Jaynes and Asan, 1976; Tuohy, 1980a,b).

Proteins may be produced from the sources mentioned above. However, unless these proteins are converted to acceptable foods or food ingredients, they will have limited value (Kinsella, 1978). The varieties of uses or potential uses of food proteins depend primarily on the physicochemical properties (or functional properties) of the proteins. Kinsella referred to functional properties to describe the composite behaviour of proteins in foods and to reflect interactions that are influenced by composition of protein, by its structure and conformation, intra- and intermolecular associations of the protein with other food components, and the nature of the medium in which these reactions may occur.

2.4.3 Effect of alkali treatment on proteins

One approach to recovering the proteins, with certain desirable technological characteristics, from both plant and animal materials is by using alkaline extraction procedures (Young and Lawrie, 1975a; Swingler and Lawrie, 1979; Jelen *et al.*, 1979). The alkali solubilizes the protein and also unfolds it to permit dispersed chains to form fibers (Lundgren, 1949). Other desirable purposes for using alkaline treatment of food ingredients include: 1) obtaining proteins with specific properties such as foaming, emulsifying or stabilizing; 2) destruction of aflatoxin in groundnuts; and 3) obtaining solutions suitable for spinning fibers (De Groot and Slump, 1969). However, alkali treatment of proteins may result in various types of chemical reactions, such as denaturation, hydrolysis of some peptides, hydrolysis of amides (Asn, Gln), hydrolysis of arginine, destruction of amino acids, β-elimination and racemization, formation of double bonds and formation of new amino acids (Fennema, 1976).

In the production of spun soy fibers, the soy proteins are first dissolved in strong alkaline solution to yield a "dope", and it is known that formation of a good fiber requires 13.5% protein dissolved in 0.95% NaOH (Ishino and Okamoto, 1975; Kelley and Pressey, 1966). Koshiyama (1971) showed that the 7S components of soy protein dissociated into a 2.5S unit at pH 11.0, and a 1.8S entity above pH 12.5. Furthermore, the subunits unfolded by exposure to alkali. However, if the buffer (0.01M glycine-NaOH) contained 0.5M NaCl, 7S component appeared unchanged up to pH 10.5. Ishino and Okamoto (1975) studied the dependence of molecular interaction in alkali-denatured protein on pH and protein concentration before and after dialysis against phosphate buffer (pH 7.2, μ=0.5). Their results showed that molecular interaction of soy protein denatured with alkali occurred above 8% concentration at pH 12.3. Under these conditions, a remarkable increase in solution viscosity or gelation was observed after dialysis against phosphate buffer, pH 7.2. On the other hand, protein treated at less than pH 11.0 and at more than 8% concentration did not increase in viscosity after dialysis. The results suggest that soy proteins change their conformation above pH 11.0. Ishino and Okamoto (1975) also observed that decomposition of cystinyl residue occurred near pH 11.0 and increased gradually at higher pH. Interaction during dialysis after alkali treatment above pH 11.0 apparently results from intermolecular bonds (hydrogen, hydrophobic, disulfide) formed by amino acid residues that become accessible to each other by conformational changes. Furthermore, at pH 7.2 and 10.4 the solution of alkali-treated proteins showed high viscosities, which appeared to be caused by aggregation and increased hydration of proteins not involving changes of the structure.

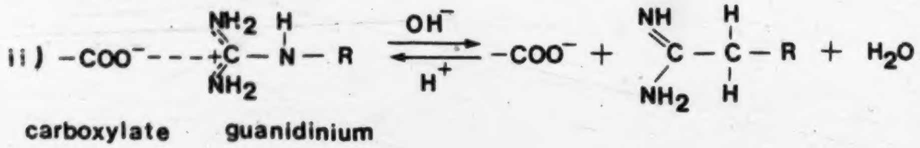
Denaturation under alkaline conditions is indicated by a decrease in the stability of the tertiary structure due to:

(a) elimination of electrostatic interactions between carboxylate and protonated amino and guanidinium groups

i) $-COO^- - - H_3N^+ \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} -COO^- + -NH_2 + H_2O$

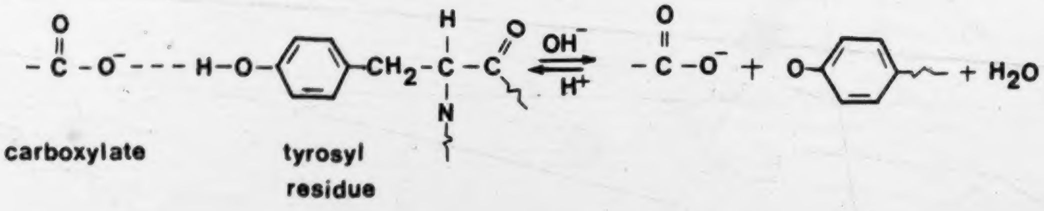
electrostatic attraction

(Glu or Asp-Lys)



$$-COO^- - - - \overset{NH_2}{\underset{NH_2}{+C}} - \overset{H}{N} - R \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} -COO^- + \overset{NH}{\underset{NH_2}{C}} - \overset{H}{\underset{H}{C}} - R + H_2O$$

(b) elimination of H-bonding between hydroxyl group of tyrosine and carboxylate groups

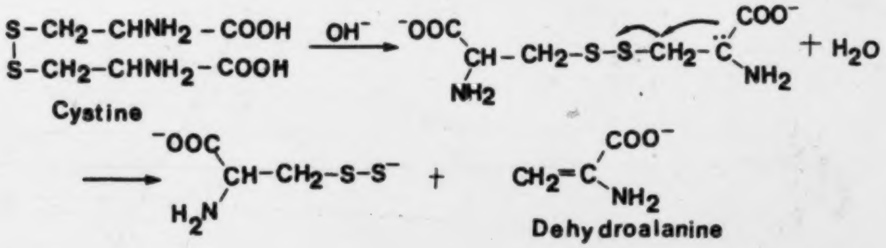


Disruption of these chemical interactions results in an increased solubilization of the protein while in the alkaline solution. However, upon adjustment to neutral or acid pH, the protein may be less soluble then originally because of denaturation.

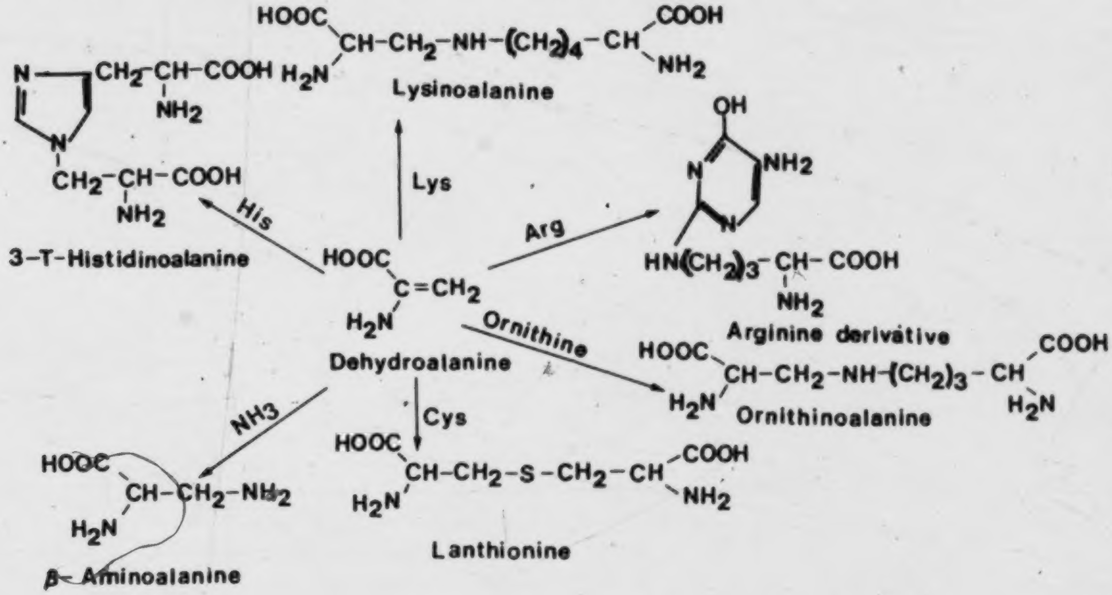
2.4.4 Formation of lysinoalanine and other new amino acids

Alkali treatment of proteins may result in formation of new amino acids wherein cysteine, lysine, arginine and possibly serine are the amino acids involved in these modifications via *β*-elimination and racemization. The *β*-elimination reaction of a cystinyl residue leads to the formation of dehydroalanine, from which various new amino acids are formed, as shown in the following reactions:

(a) formation of dehydroalanine (Fennema, 1976)



(b) formation of new amino acids (Whitaker, 1980)


Lysinoalanine
Lys
3-T-Histidinoalanine
His
Arg
Arginine derivative
Dehydroalanine
Ornithine
Ornithinoalanine
NH3
Cys
Lanthionine
β-Aminoalanine

The double bond of dehydroalanine is very sensitive with nucleophiles in the solution. These nucleophiles may be contributed by the side chains of amino acid residues, such as lysine, cysteine, histidine or tryptophan, in the protein undergoing reaction in the alkaline solution as indicated in the above reactions. For example, lysinoalanine (N -[DL-2-amino-2-carboxyethyl]-L-lysine), designated as LAL, is formed through the addition of a lysyl residue to the double bond of the dehydroalanyl residue.

De Groot and Slump (1969) noted that the amount of lysinoalanine (LAL) formed in isolated protein upon exposure at pH 12.2 increased with increasing temperature and longer exposure time. Similarly, Swingler and Lawrie (1979) found that alkaline extraction of lung and rumen at 60°C resulted in the formation of the dipeptide lysinoalanine (0.39 and

0.49 g/16 g N, respectively), with trace amounts at 20°C and above 40°C. However, Lawrence and Jelen (1982b) were not able to detect LAL in any of the samples from mechanically deboned poultry meat residues, after 1 hour alkaline extraction at room temperature or at 35 and 50°C. Samples treated for 4 hours showed measurable amounts of LAL only at pH 11.5 at 22, 35, and 50°C. After 16 hours, LAL was produced at all pH treatments at 50°C. Small amounts were also formed at 22°C and 35°C at pH 10.7 and 11.5. These authors concluded that the proposed alkali extraction procedure would not produce LAL in the protein extract under the technologically optimal conditions.

Disulfide bonds can be cleaved at an alkaline pH by treating the protein with an excess of a reagent disulfide in the presence of catalytic amounts of thiol. Smithies (1965) investigated disulfide bond cleavage and formation in proteins. He found that the cleavage products are stable and can be isolated; containing the mixed disulfide between the reagent and the exposed thiol groups of the protein. The extent of cleavage is readily controlled by the pH of the reaction, temperature, and the addition of urea. Disulfide bonds cleaved by the reaction can be re-formed by exposing the mixed disulfide of the protein to catalytic amounts of thiol. Specific side chains can be added on to the thiol groups in native proteins by treatment with reagent disulfide alone.

Donovan (1967) observed the alkaline hydrolysis of protein disulfide bonds. He reported that three sulfhydryl groups are produced for every two disulfide bonds split by hydroxide ion.

$$\begin{array}{rcl} RS\text{-}SR + OH^- & = & RS^- + RSOH \\ 2RSOH & = & RSH + RSO_2H \\ \hline 2RS\text{-}SR + 4OH^- & = & 3RS^- + RSO_2^- + 2H_2O \end{array}$$

It has been shown that this stoichiometry and kinetic behaviour is true for the hydrolysis of cystine in 2N NaOH at 60°C (Wronski, 1963). Studies of Rivett *et al.* (1965) also demonstrated that S-S fission of cystine occurs in strong alkali, but that C-S fission may take place at pH 11. Furthermore, Gawron and Odstrchel (1967) showed that C-S fission accompanied by β-elimination accounts for the hydrolysis of amino- and carboxyl-substituted cystine at 90°C near pH 10.

2.5 Meat and Poultry Proteins

Meat contains 55-78% water, 15-22% protein, 1-15% lipid and less than 4% of the total weight comprises carbohydrates, minerals, and other organic compounds. It is evident from this chemical composition that protein alone makes up 50 to over 95% of total organic solids in meat, depending on the lipid content of the muscle tissue (Goll *et al.*, 1977).

There are three major classes of muscle proteins, based on their solubilities in aqueous solvents, namely, sarcoplasmic, myofibrillar and stroma proteins. The myofibrillar proteins are the largest and are intermediate in solubility between the sarcoplasmic and stroma proteins. This protein fraction is extracted by salt solutions of high ionic strength (0.5), and includes actin, myosin, tropomyosin and troponin. In muscle, F-actin filaments, a polymerized form of G-actin, and tropomyosin and troponin, the regulatory proteins, compose the thin filaments, while the orderly aggregated myosin molecules form the thick filaments (Forrest *et al.*, 1975).

The success attained in separating the myofibrillar proteins has been attributed to the ability of these proteins to dissolve in sodium dodecylsulfate (SDS) and the separated myofibrillar proteins in SDS-polyacrylamide gels have provided a great deal of information on protein composition of the myofifbrils (Goll *et al.*, 1977). The components of myofibrillar proteins and the corresponding molecular weights, including the subunit polypeptides are presented in Table 2.2.

The general topography of the myosin molecule has been elucidated by electron microscopy of shadow-cast preparations. The model obtained from the data is that of a globular head, about 200 A in diameter by 50 Å high, attached to a rod-like tail 1300 to 1400 Å long by 20 Å wide

Table 2.2 Properties of the myofibrillar proteins.

Protein	Mol. Wt. (daltons)	Subunit Polypeptide Composition
Myosin	475,000	200,000 daltons - two 20,700 daltons - one 19,500 daltons - two 16,500 daltons - one
Actin	41,785	41,875 daltons - one
Tropomyosin	70,000	35,000 daltons - one 32,758 daltons - one
Troponin	72,000	30,503 daltons - one (TN-T) 20,864 daltons - one (TN-I) 17,846 daltons - one (TN-C)
C-protein	140,000	140,000 daltons - one
α-Actinin	206,000	103,000 daltons - two
β-Actinin	70,000	?
M-protein	160,000	160,000 daltons - one
Paramyosin	220,000	110,000 daltons - two

Taken in part from Goll et al.(1977).

(Slayter and Lowey, 1967). Myosin is composed of two heavy chains and four light chains as revealed by SDS-polyacrylamide disc electrophoresis. Tryptic digestion cleaves the myosin molecule into its subunits, heavy meromyosin (HMM) and light meromyosin (LMM). HMM is further split into S-1 and S-2 subunits while LMM is a rod of 90% α-helical content. The α-helical content of HMM, S-1 and S-2 fragments is 46%, 33%, and 87%, respectively. On the other hand, rabbit muscle G-actin is globular and in the presence of salt polymerizes into F-actin (Ebashi and Nonomura, 1973). At high salt concentration (6M KCl), actin and myosin combine to form actomyosin filaments, giving a highly viscous solution.

The organization of skeletal muscle from the gross structure to the molecular level is diagrammatically depicted in Figure 2.6. The unique structure of the striated myofibrils, composed of actin filaments (6-8 nm dia, and 1.0 um at either side of z line) and myosin filaments (14-16 nm dia, and 1.5 um long), is common to all striated muscles of vertebrates, including fish (Forrest *et al.*, 1975; Matsumoto, 1979). In longitudinal section, the thick filaments are aligned parallel to each other and are arranged in exact alignment across the entire myofibril. Similarly, the thin filaments are exactly aligned across the myofibrils, parallel to each other and to the thick filaments.

Figure 2.6 Diagram of the organization of skeletal muscle from the gross structure to the molecular level. (A) skeletal muscle, (B) a bundle of muscle fibers, (C) a muscle fiber, showing the myofibrils, (D) a myofibril, showing the sarcomere and its various bands and lines, (E) a sarcomere, showing the portion of the myofilaments in the myofibrils, (F-I) cross-sections showing arrangement of the myofilaments at various locations in the sarcomere, (J) G-actin molecules, (K) an actin filament, composed of two F-actin chains coiled about each other, (M) a myosin filament showing the head and tail regions, and (N) the light meromyosin (LMM) and heavy meromyosin (HMM) portions of the myosin molecule (Forrest et al., 1975). Note: The schematic diagram described in this figure has been removed because of the unavailability of copyright permission.

Food technologists have studied the myofibrillar protein fraction extensively because the myofibrillar proteins have a great influence on both the culinary qualities and the important technological properties of meat. The parallel orientation of the myofibrillar proteins in the intact muscle tissue imparts the characteristic texture of meat, the primary objective in simulating meat (Lugay and Kim, 1981). Further, the inherent fibrous nature and large molecular weights of myofibrillar proteins make them highly suitable in any protein texturization processes.

2.5.1 Recovery of meat waste proteins

There has been a growing interest in increasing the production of plant proteins and in the development of new sources of food proteins. The production of conventional plant proteins, like soy proteins, has been widely used in the manufacture of meat-like products. However, there are some drawbacks in using soy protein, such as low acceptability, absence of organoleptic quality, and high cost. It is highly desirable, therefore, to reassess the potential for making edible foods from the substantial amount of slaughterhouse protein which is currently wasted. Swingler and Lawrie (1979) reported that each year, in the United Kingdom alone, abattoir by-products equivalent in protein content to about 200,000 tonnes of meat fail to be utilized. If animal protein isolates could be prepared from materials which are otherwise wasted, they might compete successfully with plant protein isolates. Moreover, animal protein isolates undoubtedly have physical, nutritional or functional properties which will make them superior to those of plant origin.

Various methods have been developed to recover meat proteins from meat industry by-products. Some researchers have pursued solvent extraction of fat and dehydration as a means of protein recovery. Levin (1970) and Nash and Mathews (1971) employed carbon tetrachloride flotation in the production of meat protein concentrates and in the removal of meat residues from meat and bone meal, respectively. However, protein concentrates produced in this manner are denatured and, as a result, loss of functionality and decrease in nutritive value are common problems.

Other methods of protein recovery are based on the electrostatic properties of proteins, i.e., isolation of the proteins at electrostatic neutrality or the apparent isoelectric point. It is well established that, if a protein is subjected to either extreme of acid or alkali, then the protein will assume a net positive or negative charge, respectively (as a result of the ionization of the ionic groups). In that event, severe molecular stresses occur within a single molecule due to the repulsive forces of similarly charged groups. Such repulsion among the protein molecules will assist solubilization (Murray *et al.*, 1981).

Numerous researchers work on the alkaline side of the pH range in extracting protein from meat by-products for the manufacture of protein-based products for human consumption. Jelen *et al.* (1979) recovered protein from ground beef bones using an aqueous alkaline extraction process, at pH 10.0-10.5 and 20-22°C. The same processing conditions were employed by Lawrence (1981) in extracting protein from mechanically deboned poultry meat residues. The comminuted boning residues were agitated in an alkaline solution to loosen the meat particles and extract the proteins. The bone fragments and other solid particles were separated by centrifugation and the remaining aqueous solution acidified to isoelectric point (pH 5.0-5.3) in order to precipitate the proteins. Further investigation was conducted by Lawrence *et al.* (1982) on the technical feasibility of this alkaline extraction process. Mechanical problems relating to the particle size occurred in the alkali extraction step. However, regrinding the bone residues eliminated the blockage of pipes and pumps in the system. A similar method of recovering meat proteins remaining or which are attached to the bone after the bonning operation was disclosed by Herubel (1982), and for the production of protein concentrates and isolates from fish (Kahn *et al.*, 1975), mechanically deboned poultry meat (Young, 1975), bovine and ovine offal (Young and Lawrie, 1975), and dry meat and bone meal (Nash and Mathews, 1971).

A two-fold increase in protein yield was obtained at 60°C compared to that at 0°C (Young and Lawrie, 1974). Swingler and Lawrie (1979) found that the proportion of protein recoverable from bovine heart, kidney, liver, lung, rumen and spleen by alkaline extraction, followed by reacidification, was related to the temperature of extraction. Likewise, the recovery of both lung and rumen protein at 60°C was approximately twice that at 0°C. Extraction for more than 2 hours gave increases in protein recovery which were attributed partly to the increased solubilization of collagen. On the other hand, after solubilizing the protein from beef bones in an alkaline extract, Jelen *et al.* (1979) found that protein recovery from the extract in excess of 90% was possible by heating momentarily to 80°C or higher at pH 5.0-6.0, while lower yields were obtained at 60°C. Minimum yields were also obtained by rapid freezing at -30°C and by thawing of the pH-adjusted extracts.

Salts and polyvalent coagulants in combination with pH have been used in the recovery of proteins. A study on the influence of sodium chloride on the solubility of protein from sheep lungs showed a decrease in protein extracted at high and low pH values with increasing salt concentration, so that in general it would appear that the proteins of these tissues are most economically extracted at low ionic strength (Young and Lawrie, 1974). Adjusting the pH of abattoir effluent to 3.5 in the presence of 0.05% sodium hexametaphosphate or sodium lignosulfonate effectively coagulates the proteins (e.g., serum proteins) (Hopwood and Rosen, 1972).

The formation of lysinoalanine has been associated with the use of alkali to extract proteins, so alternative extractions have been considered to avoid the use of the basic aqueous systems. Gault and Lawrie (1980) found that anionic detergents, such as sodium dodecylsulfate (SDS), extract substantially greater quantities of protein from lungs, rumen and intestinal offal at neutral pH than does alkali at pH 10. Addition of 0.005-0.05M $FeCl_3$ precipitated the SDS-protein complex and the residual SDS removed by washing with 40% methanol or 60% acetone in the presence of 10% KCl (Ellison *et al.*, 1980).

2.5.2 Molecular weight profile of recovered meat proteins

From the texturization standpoint, the molecular weight distributions of proteins affect the morphology, microstructure, and physical and rheological properties of texturized protein products (Rhee *et al.*, 1981). For example, Rhee stressed that high molecular weight proteins (>50,000 daltons) are needed to form texture during extrusion of soy protein ingredients.

Young and Lawrie (1974) characterized the protein isolates from bovine and ovine stomach and lungs. The electrophoretic patterns of proteins from lung and stomach tissues had indicated subunit molecular weights of 13,500 to 87,000 for lung proteins and 13,500 to 145,000 for stomach proteins. Reticulum and omasum were characterized by the appearance of a component of molecular weight 28,000-28,500 giving rise to a distinct peak. These proteins were used in the preparation of spun protein fibers (Young and Lawrie, 1975a,b).

Recently, Kijowski and Niewiarowicz (1985) determined the molecular weight profile of the protein from mechanically deboned poultry meat residues, following extraction with 6% NaCl. The electrophoretic separation and identification of the proteins was performed on 8% polyacrylamide gel with sodium dodecylsulfate (SDS) after fractionation into water soluble (sarcoplasmic) and salt soluble (myofibrillar) proteins. The protein with molecular weight 48-55 x 10^3 daltons predominated among the water soluble proteins, while in the salt soluble fraction 38-47 x 10^3 and 17-21 x 10^3 dalton proteins were observed.

2.6 Functional Properties of Meat Proteins related to Texturization

The universal attempts to utilize less expensive sources of proteins to fabricate new food analogs and to develop new functional ingredients have accentuated the need for information on functional properties of proteins. Both intrinsic and applied factors influence the observed functional properties of proteins. The inherent molecular properties of the proteins *per se* (size, shape, conformation, whether native or denatured), the methods and conditions of isolation (refining, drying, storage), the degree of purification, and modification by physical (heat), chemical or enzymatic processes also influence the performance of proteins in food systems (Kinsella, 1976; Galyean and Cotterill, 1979; Cherry, 1981).

To facilitate the development of proteins with particular functional properties it is first necessary to define which physicochemical properties are most important. Kinsella (1982) suggested that a well characterized protein should be selected to be used as a model system wherein functional proteins are the major active reagents (e.g., meat proteins). When such structural and functional relationships are understood, it may be feasible to modify inexpensive proteins and impart the necessary functional properties. Kinsella defined some other chemical and/or physical attributes that are responsible for the desirable properties toward the development of functional ingredients and the fabrication of new foods. The amino acid composition, the sequence of amino acids, the manner in which secondary and tertiary folding occurs and possible associations between polypeptides all affect the final structure and physical properties of different food proteins. These structures in turn vary in their response to environmental factors (temperature, pH, ionic strength), which also affect the functional properties.

The fabrication of palatable food products is a natural progression from the isolation of recoverable protein in a concentrated form. Young and Lawrie (1974a) had shown that blood plasma can be fabricated into spun products which could prove to be acceptable meat analogues. Such results were compared to proteins isolated from porcine lung and stomach (Young and Lawrie, 1975). The findings showed that fibrous products spun from the latter protein sources appeared to have lower elasticity than those spun from concentrated blood plasma. Electrophoretic differences between the proteins from lung, stomach and plasma were also observed. The authors proposed that these differences in protein character may in part be responsible for variations in the physical properties of the spun products. However, the manner in which the protein has been treated prior to spinning may have a more appreciable effect on the fibre properties.

A number of factors may influence the mechanical properties of the spun fibers. These include protein type, mode of preparation of protein prior to spinning and conditions selected for spinning. Young and Lawrie (1975b) showed that fibrous products spun from isolated lung and stomach proteins exhibited a low resistance to shear. They also found that shear resistance of spun plasma protein was significantly affected by the extrusion rate of protein through the spinneret and by the drawing velocity of the fibers. The dope viscosity did not significantly affect the resistance of spun serum products. In an earlier study, Young and Lawrie (1975a) found that the rise in viscosity for isolates of higher protein content (120-150 mg/g) appeared to depend upon the concentration of alkali. This may be due to the presence of excessive protein aggregation. Further studies indicated that the viscosity of plasma protein, porcine lung and stomach isolated at pH 4.5 were similar and this may suggest that the state of the protein is of greater significance in determining viscosity behaviour in alkali than the particular protein type. According to these authors, this is an important factor not only because a high degree of stability of viscosity is required in spinning dopes, but also because the state of the protein in the dope may influence the mechanical properties of the resultant spun fibers.

Young and Lawrie (1975b) reported some observations of the fine structure of protein fibers as determined by electron microscopy. Using plasma protein fiber, there was no orientation of protein molecules along the axis of the fiber. Observation of an electron micrograph of a cross-section of normal serum protein fiber indicates that the fiber consists of spherical protein particles linked together in chains or strands to form a three-dimensional mesh incorporating pores of varying sizes. More random aggregation in some regions was also reported to be evident. In the fiber of high shear resistance, it was reported that the gel structure of the fiber was more clearly defined and the protein strands easily discernible. A similar structure was apparent in the fiber of medium shear resistance, but in this case the three-dimensionally linked chains were not as obvious and some fracturing of the system appeared to have taken place. The fiber of low shear resistance was not structurally well-defined and a higher degree of random aggregation of protein had occurred.

Observations on other protein isolates were also made (Young and Lawrie, 1975b). For example, fibers spun from lung protein isolate exhibited a markedly different cross-sectional appearance than those spun from plasma protein. There was minimal evidence of specifically oriented aggregation of protein to form a gel structure. Randomly aggregated protein predominated and the pores between the protein aggregates were of much larger dimensions than those occurring in the plasma protein fibers. In the case of stomach protein fibers, the structure was more closely related to a gel and dimensions of the pores betwen the strands were relatively large.

The information on fiber structure gained from the electron microscopy studies could be correlated to some extent with the texture analysis. Poorly defined structures and large pores found in the fiber of lung and stomach proteins give rise to low mechanical strengths. The inferior structure of these fibers may reflect the degree of protein aggregation occurring during treatment prior to spinning, which differs from that applied to plasma protein. Alternatively, the nature of protein constituting the lung and stomach extracts may be such that the opportunity for oriented interactions is minimized due to the absence of the groups required for such linkages. Further studies by Young and Lawrie (1974b) revealed that the molecular weights of the proteins strongly influence the mechanical properties of the resultant spun fibers. It was clearly demonstrated electrophoretically that low molecular weight components (13,500 to 87,000) were present in lung protein and higher molecular weights (13,500 to 145,000) were found in the major portion of stomach protein. Higher resistance to shear and more oriented gel structure in stomach than in lung protein fibers may be attributed solely to protein composition.

2.6.1 Freezing and frozen storage of myofibrillar proteins

Since the main protein type used in the present freeze texturization studies was myofibrillar proteins, this section will focus on the various investigations of the behaviour of these proteins (myosin, actin, actomyosin) at zero or sub-zero temperatures. The environmental conditions to which the myofibrillar proteins were subjected during the freezing and frozen material studies discussed in this review might be entirely different from the conditions during freeze texturization of meat protein isolates. Nevertheless, the preceding discussion should provide a greater insight on the nature of reactions that might occur with myofibrillar proteins under freeze texturization conditions.

2.6.1.1 Behaviour of myofibrillar proteins during freezing and frozen storage

The separation of water as pure ice in frozen muscle has two main consequences. One, the protein molecules are brought into close contact and are subjected to an environment which contains a greater concentration of low molecular weight substances present in the unfrozen muscle. The tissue salts that are present in high concentrations in frozen fish denature the proteins (Connell, 1962). The results had indicated that, at a temperature just below the freezing point of the fish, the rate at which certain protein changes occurs increases as the temperature of storage is lowered, until about -1 to -3°C is reached, and then decreases. As the temperature of storage is reduced, more water is frozen out and the concentration of salts in the unfrozen part increases. Therefore, on the assumption that greater concentration of salt causes more protein damage over the same period of contact, damage will occur as the temperature is lowered. However, below a certain temperature the inverse effect on the rate of reaction predominates and the rate of protein damage falls. Two, the finding that the rate of initial freezing affects the storage can be accounted for on the grounds that a particular configuration of ice crystals may give rise to especially high local concentrations of salts.

Khan (1966) found a number of changes occurring in muscle protein fractions during frozen storage, including loss of solubility, ATPase activity and sulfhydryl content of the myofibrillar proteins.

Monodisperse cod myosin has a pronounced tendency to aggregate when neutral solutions are left undisturbed at 0°C (Connell, 1959). The aggregation is characterized by the appearance in the ultracentrifugal picture of the solution of at least one, and sometimes two, discrete components which sediment faster than actomyosin and which, on the basis of their sedimentation coefficients, can be identified as a side-to-side dimer and trimer, respectively. The rate of aggregation is greatly affected by the temperature of storage and eutectic temperature (Connell, 1959; Snow, 1950). Further, Connell attempted to elucidate the mechanism of aggregation of cod myosin. He stated that aggregation occurs without appreciable intramolecular change because the specific rotation and numbers of sulfhydryl groups titratable with N-ethylmaleimide (NEMI) do not change during the first 7 days storage at -7°C. The aggregation of cod myosin in the unfrozen state at 0°C apparently does not proceed by a conversion of SH groups to SS bridges because (a) the aggregation is not inhibited by the addition of 0.01 1M monothioglycol and (b) myosin in which the reactive SH groups of which have been blocked by NEMI aggregates just as rapidly as untreated mysosin.

The behaviour of heavy meromyosin (HMM) and light meromyosin (LMM) during freezing and storage at -20°C was investigated by Matsumoto *et al.* (1977). The following summarizes the results obtained in this study: 1) there were no significant changes in solubility curves of HMM and LMM;

2) the ATPase activity of HMM decreased to 50% of the pre-freezing value after one day and was absent after 2 weeks storage; 3) the ability of HMM to bind with F-actin, as determined by electron microscopy, was lost after 2 weeks frozen storage; and 4) LMM, after dialysis against a solution of 0.05M KCl-0.005M tris-maleate buffer (pH 6.2), also exhibited a decreased capacity to form well-ordered paracrystals as examined by electron microscopy. Matsumoto (1979) stated that these results may indicate that denaturation of myosin occurs both in HMM and LMM segments. Further, he suggested that the decrease in the ATPase activity and F-actin binding capacity of HMM may indicate conformational change in the light chains which are located in the S-1 region of HMM. Some arguments were presented against the possiblity of a conformational change of myosin molecules during frozen storage, based on the lack of an observed change in the specific rotation (Connell, 1959). However, Matsumoto (1979) stated that a conformational change in the light chains of HMM may not necessarily be acompanied by an appreciable change in specific rotation. A change from an ordered random coil to a disordered random coil appears to have occurred during frozen storage of HMM and myosin, thus, an appreciable change in specific rotation would not be expected. He further demonstrated denaturation of isolated carp actin during frozen storage at -20°C. The loss of the polymerizing ability of G-actin molecules suggested that the conformation of the native globular

G-actin must have been impaired during frozen storage.

The change in amount of soluble actomyosin is regarded as the primary criterion of freeze denaturation. However, it should be noted that the solubility data do not tell precisely how much protein is denatured and how much is native; rather they provided a relative measure of denaturation (Matsumoto, 1979). This author also found a decrease in solubility in frozen storage experiments with either intact muscle, protein solution or with suspension of isolated actomyosin. Further, the viscosity of soluble actomyosin fractions decreased with increasing time of storage, suggesting that the actomyosin had become less rod-like or filamentous either by individual folding or by aggregation of the filaments. The ultracentrifugal analysis of the soluble fractions showed that the actomyosin peaks (20S-30S) decreased in urea, while several faster moving peaks simultaneously appeared with increasing time of frozen storage. Based on these results, it has been proposed that actomyosin forms various aggregated states during frozen storage, which is in agreement with the viscosity change (Suzuki *et al.*, 1964; Oguni *et al.*, 1975; Noguchi and Matsumoto, 1970).

2.6.1.2 Mechanism of aggregation of fish actomyosin

Based on some hydrodynamic properties and analysis by electron microscopy, Oguni *et al.* (1975) and Matsumoto (1979) proposed the mechanism of aggregation of fish actomyosin during frozen storage as depicted in Figure 2.7.

Figure 2.7 Proposed mechanism of the aggregation of fish actomyosin during frozen storage. Formation of network aggregates through: (1) aggregation of F-actomyosin and (2) dissociation of F-actomyosin (Matsumoto, 1979). Note: The schematic diagram described in this figure has been removed because of the unavailability of copyright permission.

Electron microscopic studies of isolated preparations of fish actomyosin denatured by frozen storage showed that actomyosin filaments with arrowhead structures aggregated side-to-side and cross-wise when thawed immediately after freezing. As time of frozen storage is increased, further aggregation formed network structures (Oguni *et al.*, 1975; Tsuchuiya *et al.*, 1975; Ohnishi *et al.*, 1978). In addition to aggregation, dissociation of F-actomyosin into F-actin and myosin occurred. It was suggested that the dissociated F-actin, as thin filaments, became entangled and aggregated and that the dissociated myosin monomers folded into globular form. At advanced stages of freeze denaturation, large masses with diffused outlines were frequently found, suggesting complex aggregation of actin and myosin (Matsumoto, 1979). Further, from Figure 2.7, it was suggested that the dissociation of actomyosin into actin and myosin could be due to a shift in the equilibrium, actomyosin $\rightleftharpoons$ actin + myosin, by the high concentrated salt solution of the unfrozen liquid portion in the protein-water system (Hamoir, 1955; Ellis and Winchester, 1958). However, Matsumoto (1979) argued that, if this is true, the dissociated actin and myosin must re-associate immediately after thawing. Matsumoto pointed out that this may be difficult since the ability to associate is decreased during frozen storage.

Kinetic studies on the denaturation mechanism of carp actomyosin during frozen storage were conducted by Ohnishi *et al.* (1978). The results showed that the solubility decrease proceeded not as a single first order rate but in two or more successive stages, each having a first order expression. The rate of solubility decrease was not significantly different when protein concentration and ionic strength were varied. At a pH above 7.5, the result was further complicated by the appearance of three or four successive stages. At a pH below 6.2, the rate of the first stage was very high. Electron microscopic analysis of the frozen stored samples at neutral pH showed that, during frozen storage (-20°C), actomyosin filaments enfold and aggregate with each other, the arrowheads of the filaments becoming vague and then being released. This might be attributed to the decreased affinity between myosin and actin. Ohnishi *et al.* (1978) also noted that at pH 5.8 and 9.4, the actomyosin filaments, even before freezing, were deformed as compared to the normal filaments at neutral pH. They suggested that this result indicates the denaturing effect of storage acidity and alkalinity, respectively.

Buttkus (1970, 1974) proposed that denaturation of muscle proteins in frozen storage is caused by the formation of disulfide bonds between protein molecules, while Matsumoto (1972) showed that various intermolecular bonds, i.e., ionic, hydrogen, disulfide and non-polar, contribute to the denaturation of protein.

Fennema (1982) discussed the changes in protein structure at low temperature, summarized from various studies. He stated that, among the chemical bonds that hold the tertiary native structure of protein, hydrophobic interactions are considered as being of primary importance in most proteins, and the strength of these interactions is weakened by lowering the temperature. Taborsky (1979) stated that the tendency of the proteins to engage in hydrophobic interactions can be considered largely as an entropy-driven process. The unfavorable low entropy of "ordered" water, which surrounds nonpolar groups when they are exposed to the aqueous medium, is avoided because the nonpolar groups fold inward. This is accompanied by an entropy-increasing release of ordered water to the relatively disordered state of the bulk solvent. He further stated that considering that bulk water itself becomes more extensively hydrogen bonded and thus more highly ordered as temperature is lowered, the entropic advantage of folding nonpolar groups inward is lessened at low temperature. Thus, lowering the temperature will be critical in those proteins that are stabilized by hydrophobic interactions.

Connell (1962) stated that, if cross-linking in frozen stored fish is a consequence of the interaction of denatured proteins, it would be expected that the kind of bonds formed would be similar to those occurring between proteins denatured and aggregated in solutions. There are good reasons for believing that the latter are of a secondary type formed between the apolar regions of adjacent molecules. These regions are normally buried in the interior of the molecule but are brought to the surface as a result of denaturation. Experiments showed that the large majority of cross-links formed during the frozen storage of cod are secondary in character in the sense that they are capable of being split by concentrated solutions of sodium dodecyl sulfate. The secondary types of bond under consideration here are hydrogen bonds, hydrophobic bonds and bonds resulting from van der Waals dispersion forces. However, Fennema (1982) emphasized that the simple relationship between hydrophobic interactions and temperature is not capable of accounting for all protein structural changes observed at low temperatures. Other factors, such as pH, ionic strength, surface tension, protein concentration, and concentration of nonprotein solutes change substantially during freezing and have profound effects on the structures of proteins.

Low temperatures should enhance hydrogen bond formation due to the negative change in enthalpy that characterizes this process (Taborsky, 1979). Therefore, it would be expected that hydrogen bonds become stronger as the temperature is lowered and such strengthening should apply to intramolecular bonds (between donor and acceptor groups of the protein) as well as to the intermolecular bonds (between protein groups and water molecules).

2.6.2 Cheese whey proteins

Whey is a dilute fluid resulting from the conversion of bovine milk into cheese. Whey contains about 6.5 g/L solids, of which the major constituents are lactose (70-80%) and protein (9%). In general, there are two types of whey: sweet whey from the manufacture of cheese or casein from milk by the action of rennet-type enzymes with relatively little or no acidity development during the milk clotting; and acid whey where the milk is coagulated primarily with acid. Sweet whey has a minimum pH of 5.6, while acid whey has a maximum pH of 5.1 (minimum about 4.0) (Marshall, 1982; Evans and Gordon, 1980).

The major protein constituents of whey are β-lactoglobulin, α-lactalbumin, bovine serum albumin, the immunoglobulins and proteose-peptones. Of these proteins, β-lactoglobulin and α-lactalbumin are present in the highest concentration and are probably of primary importance in the physicochemical properties of whey protein products. Other proteins are present in whey, including lactoferrin, lactollin, glycoprotein and blood transferrin. The whey proteins are mainly large globular proteins. β-lactoglobulin, which comprises 50% of the proteins, has a monomer molecular weight of 16,300 daltons but exists as a dimer between pH 3.5 and 7.5. The immunoglobulins are a mixture of glycoproteins of different sizes and are grouped together because they have a common antibody activity. The proteose-peptone fraction contains a mixture of glycoproteins and phosphoproteins with a range of molecular weights. A significant number of sulfhydryl groups are also present in the major whey proteins. β-lactoglobulin, α-lactalbumin and bovine serum albumin contain 1.6%, 1.9% and 1.9% sulfur, respectively (Marshal, 1982; Schmidt and Morris, 1984; Robinson *et al.*, 1976).

2.6.3 Properties of whey proteins

The undenatured or native whey proteins are soluble at all pH values, a factor which distinguishes them from the caseins. Except for the stable proteose-peptone fraction, whey proteins are sensitive to temperatures above 60°C, the degree of denaturation depending on the protein component, total protein and solid concentrations, pH, ionic strength, temperature and time of exposure. They undergo denaturation due to unfolding of their compact globular conformations, ultimately to a random conformation. At certain degrees of unfolding, denaturation may be accompanied by aggregation as a result of hydrophobic interactions and intermolecular disulfide interchange. Whey proteins may form gels or precipitates according to the condition of denaturation. The extent of whey protein insolubilization or denaturation is usually denoted by the loss of solubility at pH 4.6-5.0 and can be monitored by gel electrophoresis. Whey proteins are sensitive to various denaturants such as heat, alcohol, and other polar organic molecules (Evans and Gordon, 1980; Morr, 1974).

2.6.4 Recovery of whey proteins by heat precipitation

The concept of protein denaturation is sometimes confused with aggregation or coagulation. Conditions which promote protein denaturation do not necessarily encourage protein aggregation. The aggregation of whey proteins by heat, heat-acid, urea or alkali is preceded by denaturation and may be followed by coagulation and precipitation (Hill *et al.*, 1982a). Heat-denatured whey proteins are structurally altered to expose sulfide groups. These structural changes are rapid at pH's greater than 6.7 and temperatures greater than 70°C (Lyster, 1972). Sawyer (1968) demonstrated that aggregation follows primary and secondary reactions. The primary products are colloidal aggregates formed by SH/SS interchange reactions at temperatures greater than 70°C, while the secondary reaction, which is favored at 60-70°C, yields larger colloidal structures formed by nonspecific aggregation of the primary products. This secondary reaction is not dependent on SS bonding and its products may undergo further nonspecific aggregation, yielding a precipitate coagulum. Another type of nonspecific primary aggregation takes place if SH groups are blocked by N-ethylmaleimide, but this pathway results in lower yields of secondary products.

Several studies have been reported dealing with the effect of calcium ions on the heat aggregation of whey proteins. Townsend and Gyuricsek (1974) showed that Ca^{++} highly promoted the heat aggregation of whey proteins at pH above 6.0. DeWit (1981) noted that the amount of Ca^{++} required to induce the aggregation of β-lactoglobulin was equivalent to its net negative charge. Zittle *et al.* (1957) suggested that Ca^{++} binding neutralized the net negative charges of whey proteins and caused isoelectric precipitation. On the other hand, Varunsatian *et al.* (1983) compared the effects of Ca^{++} on the heat aggregation of whey protein concentrate with those of Na^{+} and Mg^{++}. They found that, on the alkaline side of the isoelectric zone, aggregation of the whey protein concentrate (WPC) was increased by the addition of $CaCl_2$, $MgCl_2$ or NaCl, among which $CaCl_2$ showed the greatest effect.

The term "heat-acid precipitation" means that heat-denatured colloidal proteins are destabilized by the addition of acid in the presence of calcium. Isoelectric precipitation may also be induced by addition of calcium, but cheese ash content is increased and bitterness may result. Generally, the recovery of protein and the composition of heat-precipitated whey protein (HPWP) depend on pH, temperature and ionic strength. The optimum pH depends on the type of whey and is probably related to the initial pH and calcium content of the whey (Robinson *et al.*, 1976). These authors obtained maximum protein precipitation from sweet whey (high pH, low calcium) by acidifying to pH 4.5 before heating, and from acid whey (low pH, high calcium) by neutralizing to pH 6.5 with sodium hydroxide before heat treatment. Panzer *et al.* (1976) affirmed that protein recovery from sweet whey is maximized by heat denaturation at about pH 6.5, followed by acidification and/or addition of calcium to induce gross aggregation. In the case of acid whey, neutralization before heating aids protein denaturation, but the ionic conditions are such that subsequent acidification does not affect the aggregation process except to lower the HPWP ash content.

Hidalgo and Gamper (1977) studied the precipitation of rennet whey protein. In the absence of calcium chloride, maximum precipitation of protein was induced by heating at pH 6.6-6.9, cooling to room temperature and adjustment of pH to the isoelectric zone. In the presence of 0.03M $CaCl_2$, the aggregation of whey after heating and cooling was independent of pH over a pH range of 2-12. The interaction effects of pH and ionic strength may account for the different acid and heat treatments which have been recommended for the recovery of proteins from whey. In addition, the mechanics of processing are also important. For example, Hill *et al.* (1982b) reported that under laboratory conditions maximum protein recovery was obtained from sweet whey by heating without adding acid. However, in the pilot plant, acid addition after heating was required in order to induce the formation of larger curd particles and to facilitate separation by filtration on a wire screen.

The theoretical maximum recovery of crude protein (total N x 6.4) from whey is 55-65% because the heat-stable proteose-peptone fraction and non-protein nitrogen (NPN) constitutes 35-45% of whey nitrogen (Larson and Rolleri, 1955). Commercially feasible processes should recover at least 50% of the crude protein.

Panzer *et al*. (1976) described a continuous process for recovering cottage cheese whey involving neutralization to pH 6.0, heating at 120°C and an optional reacidification to pH 4.6. Reacidification decreased the ash content of the HPWP from about 23% to less than 5%. Ash was further reduced to 2% by washing the precipitate with water. On the other hand, Modler and Emmons (1977) obtained 85-97% recovery of protein nitrogen from condensed whey (17.5% solids) by acidifying to pH 2.5 and heating to 95°C. However, when single strength whey was subjected to the same process, little or no precipitation occurred. Knipers (1975) also reported that single strength whey may be acidified to pH 2.7-3.3 and subsequently heat sterilized without precipitating the whey protein. Shulkamy and Monib (1976) obtained maximum precipitation of protein from sweet whey by acidifying to pH 3.5 and heating to 95°C for 20 min.

2.6.5 Chemical precipitation of whey proteins

The separation of whey proteins which retain native properties (solubility and the ability to gel, whip or emulsify) requires the use of chemical precipitants such as polyphosphates. Hartman and Swanson (1966) recovered practically all proteins from whey at pH 2.5 with 0.5% sodium hexametaphosphate (HMP) in the absence of heat. The precipitated proteins gave electrophoretic patterns similar to the native protein. Hidalgo *et al.* (1973) obtained 90% recovery of protein nitrogen by acidifying to pH 3.0 and adding 0.1 and 0.7% HMP to acid and sweet whey, respectively. Protein recovery was improved by demineralization prior to precipitation and the residual HMP was reduced by ion exchange or gel filtration. The final product showed some native properties and contained only 9.9% residual HMP, 88.3% protein and 1.8% lactose.

2.6.6 Ultrafiltration of whey

Concentration of whey proteins by ultrafiltration technique is a comparatively new process. The physical and chemical conditions are relatively mild and little denaturation of the protein takes place. The principle of the process is based on the ability of various polymeric membranes to discriminate between molecules on the basis of their molecular shape, size and, to some extent, charge (Lewis, 1982).

Ryder (1980) reported the economics of utilizing whey by ultrafiltration, and this can be concentrated by a factor up to 20 times. The concentration factor is a measure of the extent of an ultrafiltration process. It is defined as the ratio of the volume of original feed to volume of final concentrate. This concentrate can be evaporated under vacuum and spray dried to produce a concentrate containing up to 65% protein. The composition of whey protein concentrate prepared by ultrafiltration can be varied by changing the concentration levels, by adding water at an appropriate stage to alter the protein/lactose/ash ratios (a process referred to as diafiltration), by choosing various wheys as starting material, or by altering the pH for processing (Delaney and Donnelly, 1970).

The quality of the separation or concentration achieved in an ultrafiltration process will depend upon the characteristics of the selected membrane (Lewis, 1982). The desirable characteristics for such a membrane include: a) a uniform pore size (sharp molecular weight cut-off value); b) high permeability; c) cheapness and reproducibility; and d) resistance to a wide range of chemicals, including solvents, acids, alkalis, and sanitizing agents. Furthermore, it should not react with or physically alter the proteins being processed.

Hill and Hansen (1964) identified the individual proteins in WPC by using polyacrylamide gel electrophoresis. The WPC prepared by ultrafiltration contains 68.4% β-lactoglobulin, 21.3% α-lactalbumin and 10.3% serum proteins. This finding is approximately the same ratio of the proteins present in whole milk. They concluded that the ratio of individual proteins in any ultrafiltered WPC will be influenced by such factors as: a) type and source of whey; b) rejection characteristics of ultrafiltration membrane employed; and c) degree of concentration achieved. Delaney *et al.* (1972) observed that, owing to the mild operating conditions during concentration and drying, the protein undergoes little denaturation and the powders are very soluble. Such high protein powders possess useful functional properties and can be incorporated into a wide variety of formulated foods (International Dairy Federation, 1979).

The other methods of recovering whey proteins include reverse osmosis, electrodialysis, ion exchange resin treatments and gel filtration by the basket centrifuge technique (Hill *et al.*, 1982b).

2.6.7 Functional properties of whey proteins related to texturization

As mentioned elsewhere, commercially available textured protein products are manufactured from oilseed proteins, mainly soybean. These products are being used in increasing amounts as ingredients in the processed meat industry. Whey proteins are nutritionally superior to the oilseed proteins in commercial texturized vegetable protein (TVP) products (Tuohy, 1980).

Most experimental work on the texturizing of milk proteins has concentrated on the production of meat-like fibers from casein by wet spinning (Boyer, 1953; Downey and Burgess, 1979a; Downey and Burgess, 1979b). Whey protein concentrate has also been used as raw material (Jaynes and Asan, 1976; Asan, 1974), who evaluated the formation of fibers from WPC using a wet spinning process. Continuous fibers with good handling properties were formed from 12%

and 14% aqueous alkaline solutions of WPC/detergent containing equal concentrations (on WPC weight) of sodium dodecyl (lauryl) sulphonate (SDS) or sodium dodecyl benzene sulphonate (SDBS) and mercaptoethanol. Fibers were spun into an acetic acid-sodium chloride bath. The detergent was extracted with 60% aqueous acetone with or without added potassium chloride. The fibers contained about 95% protein and were not affected by boiling in water for 30 min.

To date, little work has been done on the feasibility of texturizing milk proteins by expansion methods, for use as meat extenders. Tuohy (1979) reported that conventional thermoplastic equipment was unsuitable for texturizing skim milk protein and whey protein. Also, Tuohy (1980a) reported the use of a modified twin-screw extruder; skim milk protein was successfully texturized, but not whey protein.

A comparison of the texture of expanded milk protein meat extenders and TVP was made by Tuohy (1980a), who found that the texture of extruded skim milk protein did not resemble closely that of minced beef or commercial TVP. Tuohy also reported that an alternative thermoplastic method was developed to texturize milk proteins by Poznansk *et al.* (1977), yielding a product with a structure resembling meat tissue. However, no further information has been published on the texture of this product.

In another study, Tuohy (1980b) produced a textured whey concentrate in aqueous solution (a dope) with microwaves at a frequency of 2,450 mc. Texture was evaluated using the Minnesota Texture Method (M.T.M.). Three texture parameters -- 1) minimum compression-shear-extrusion force, 2) energy required for compression-shear-extrusion, and 3) slope of the compression force displacement line -- were used to measure the effect on texture of altering the pH, protein/dry matter content and the casein/total protein ratio of the dope. Minimum texture values were found at pH 5 and maximum at pH 7-9. An increase in the protein/dry matter content of the dope from 45-65% increased the maximum force from 62 kg to 243 kg. When casein replaced 50% of the whey protein, the maximum force fell from 168 to 51 kg. Tuohy further added that similar changes were found in the other two texture parameters.

Ozimek *et al.* (1981) studied the influence of fat content and added salt on the water absorption capacity of textured milk proteins. Variation in fat content of the product (11% and 15%) was obtained by introducing various amounts of fat to skim milk during the texturization process. The salt concentration of the preparation was modified by adding sodium chloride, Hamine polyphosphate mixture or both. The results showed an inverse relationship between the level of fat content in the product and the amount of water absorbed. This indicated that the process of water absorption in the textured protein was limited and inhibited by fat being hydrophobic. The preparation to which no salt had been added was characterized by the lowest absorption capacity. This might have been due to the effect of sodium chloride on proteins. For example, in the case of muscle proteins, the sodium and chloride ions become bound to the polar protein groups with opposite charges (Hamm, 1973; Hermansson and Akesson, 1975). Initially, chloride ions are bound to the protein, thus, the "net protein charge" of the protein molecules increases. Subsequently, this results in loosening of the protein network, making it possible for water to enter (Hamm, 1972).

There has been increasing interest concerning the physicochemical properties of whey proteins, with respect to the use of these proteins as primary raw materials or ingredients in food products. However, few studies have been conducted on whey proteins in regard to texturization. No work has been done to date on the feasibility of texturizing whey proteins by freezing.

2.7 Nutritional Aspects of Texturized Protein Products

Textured protein products have found increasingly greater usage in typical American diets since the early pioneering work of John Harvey Kellogg (Kinsella, 1978). Several methods have been developed for producing these food products with desirable palatability characteristics and at a competitive cost. The consumer acceptance of textured protein products has been extended to meet both economic affordability and nutritional expectations of the general public.

The nutritional standard for a textured protein product should be that of the nutritional value of the traditional product it resembles, for example, meat, which is considered an important source of nutrients such as protein, thiamin, riboflavin, vitamin B_{12} and the minerals iron and zinc (Kies, 1974). The Food Standards Committee (United Kingdom) has recommended some nutritional qualities for textured vegetable protein (TVP) as meat extenders or replacers (Table 2.3).

Table 2.3. Some recommendations for the nutritional quality of textured vegetable proteins as meat extenders or replacers in the UK.

Composition	Amount
Protein	$\nless$ 50% (on dry weight basis)
Methionine	$\nless$ 2.6 g (per 100 g protein)
Vitamins	$\nless$ 2.0 mg thiamin (per 100 g dry matter) 0.8-1.6 mg riboflavin 5.0-10.0 μg vitamin B_{12} 10.0-20.0 mg zinc

From Richardson (1982).

Most vegetable protein foods have a lower biological value for the protein they contain than muscle meat, egg protein and casein, when evaluated in animal diets (Richardson, 1982). This nutritional problem has been attributed to methionine, the limiting amino acid in vegetable proteins. However, this problem could be rectified through addition of the sulphur-containing amino acid to the

TVP food products. Wormack *et al.* (1974) demonstrated the differences in the availability of amino acids in textured soy, soy isolate, and soy isolate supplemented with methionine (Table 2.4). The bioavailability of individual essential acids was estimated using the protein efficiency ratio (PER) method.

Table 2.4. Protein efficiency ratio (PER)[1] of soy products[2].

Textured soy	2.34
Soy isolate	0.91
Soy isolate + methionine	2.54

[1] casein = 2.50
[2] from Wormack *et al.* (1974).

This test shows that the sulfur-containing amino acids in soy isolate were less available than those in the textured soy. Sulfur amino acid contents of the two products were similar. In a separate study, the authors also found that lysine, threonine, tryptophan, histidine, and leucine were significantly less available in the soy isolate than in the textured soy.

Doraiswamy (1972) conducted a human bioassay study to determine the protein value of three textured protein products. An extruded, defatted soy flour product, a spun, concentrated soy product, and a wheat protein product were tested against ground beef and whole egg. Results showed no significant differences in protein value when determined between the three test products, but all gave significantly poorer nitrogen balances than did beef or egg. Other controlled human feeding studies showed TVP below the nutritional value when compared to meat, on the basis of nitrogen balance data (Kies, 1974; Poullain *et al.*, 1972). Again, DL-methionine supplementation of the TVP products was effective in improving the nitrogen balance. On the other hand, comparison of the protein value of milk and soybean isolates showed no significant difference when 2 grams of protein per kg body weight per day were fed. But when the dietary protein was decreased to less than optimal amount (a critical test level), 138 mg of nitrogen from textured protein product was required as compared with 97 mg from milk per day to maintain the nitrogen balance (Bressani *et al.*, 1967). In animal feeding studies, these authors found that rats indicated a protein value of 90% that of casein and of dehydrated beef, when fed with textured soy protein with the addition of egg albumin and wheat gluten. In dogs, the nutritive value of the textured protein product and ground beef were the same.

Debry *et al.* (1974) investigated the acceptability of textured soy proteins flavoured with ham or beef, by adults and children. The children's daily diets contained 18 g dried textured soy protein (20% total daily protein). Four kinds of diets were used successively: fixed normal diet without soy protein for 1 week; fixed diet containing soy protein for 1 week; *ad libitum* diet without soy protein for 1 week; and *ad libitum* diet containing soy protein for 1 week. The results showed no significant differences between food intakes during the fixed diets and those of the *ad libitum* diets with or without soy protein. In implementing school lunch programs using textured protein products in combination with ground meat, the USDA Food and Nutrition Notice 219 (USDA, 1971) specified the following requirements: 1) a minimum of 50% protein; 2) a maximum of 30% fat; and 3) fortification with specific levels of two minerals and six vitamins.

The wide acceptance of TVP in the human diet has been hindered by some problems inherent in the raw materials. Satterlee (1981) reported that, when a diet containing textured protein foods of the soy protein isolate type was fed to a group of human subjects, all remained in good health except that a few reported subjective symptoms of abdominal gas pains. In contrast, Kies and Fox (1971) reported no complaints of flatulence from subjects consuming 25 and 50 g textured soya flour daily. Although not strictly posing a nutritional problem, the flatulent nature of soybean may restrict its utilization in the production of textured food products. The flatulence-causing factors are the low molecular weight carbohydrates, the oligosaccharides (raffinose and stachyose), that are found at significant levels in defatted soya flour, though to a decreasing extent in full-fat soya flour, soya protein concentrates and soya isolates. Flatus production is caused by the anaerobic fermentation of the oligosaccharides in the lower intestines.

A variety of protein sources have been found suitable in other texturizing techniques. Nutritionally, though, the fibers spun from blood plasma tend to have relatively low titres for isoleucine and methionine. However, judicious mixing of proteins from various offal sources permits the spinning of fibers which have excellent amino acid content (Young and Lawrie, 1975a). Swingler *et al*. (1978) further reported that most of the lysine in the fibers appears to be available. Based on the rat feeding trials, the spinning of fibers enhances the net protein utilization (NPU) in comparison with the original protein isolates, despite a slight lowering of the methionine content. It was concluded that in preparing the fibers some inhibitors, originally present in the isolates, are removed or destroyed.

2.7.1 Effect of alkali treatment on the nutritional quality of textured proteins

Alkali treatment has been widely used in food processing due to its ability to solubilize and to isolate proteins (Tannembaum *et al*., 1970). For example, in preparing protein from meat deboning residues, alkaline treatment is needed to loosen the meat particles followed by solubilization of the proteins (Lawrence, 1981; Young and Lawrie, 1975), suitable for texturization (Wipf, 1972). Improvement of some functional properties, such as foaming and emulsifying capacities, is also achieved (Circle and

Smith, 1972). In addition to the favorable technological properties imparted to foods by alkali treatment, it may have either positive or negative effects on the nutritive value of the resulting products, depending upon such factors as the nutrient in question, characteristics of the raw materials, and extent of alkali treatment. Various undesirable chemical changes may occur, varying in type and degree with the intensity of the treatment, viz. the degree of alkalinity, temperature, and duration of exposure. The main types of change are racemization and destruction of amino acids, splitting of peptide bonds, and formation of new amino acids. The latter include lysinoalanine (Bohak, 1964), lanthionine (Hupf and Springer, 1971), and ornithinoalanine (Ziegler *et al.*, 1967). Of these three, lysinoalanine has received the most attention, probably because of the relatively easy detection and frequent presence in several types of treated proteins. The LAL cross-links, formed by the addition of lysyl residue to the double bond of a dihydroalanyl residue, decrease the number of enzyme-specific peptide bonds that can be cleaved and also serve as a steric hindrance to the proteolytic enzymes trying to cleave the amino acid residues further down the chain, both of which result in a decrease in protein digestibility (Satterlee and Chang, 1982).

2.7.1.1 Nutritional deficiency arising from alkali-treated proteins

There is no doubt that several of the changes which occur in proteins under drastic conditions of treatment may lead to a reduction in protein quality. This effect is illustrated in the results obtained by De Groot *et al.* (1977) on soy protein isolated after alkali treatment under different conditions of pH, temperature and duration. The treated protein was precipitated at pH 4.5, and examined by amino acid analysis and by protein quality assays in rats. Treatment at 40°C for 4 hr, at increasing pH levels, did not result in significant changes in the parameters examined up to pH 10. However, under these conditions detectable levels of LAL were noted. A further increase of pH to 12 resulted in a marked loss of cystine, accompanied by a sharp decline in NPU and considerable increase of LAL. At higher temperatures (60 and 80°C), there was a distinct loss of serine.

Previous studies on the effect of alkali treatment on isolated soy protein indicated that the destruction of cystine or lysine in proteins often means decreased nutritive value because the majority of food proteins are limited by either the sulfur-containing amino acids or lysine (De Groot and Slump, 1969). It was concluded that the extent of impairment of protein quality was distinctly correlated with the severity of alkali treatment. Struthers *et al.* (1979) reported that the PER for diets supplemented with 3,000 ppm LAL (30% alkali-treated soy protein) was 1.8, compared to 2.8 for the untreated soy protein control diet.

The lower PER value of the alkali-treated soy protein was due to poor digestibility of the cross-linked proteins.

Whitaker (1980) reported that the nitrogen digestibility values of 0.2M and 0.5M NaOH-treated casein (80°C, 1 hr), as determined in rats, were 71 and 47%, respectively, compared to 90% for untreated casein. He also cited that severely alkali-treated casein fed to dogs was eliminated unchanged in the feces, indicating that it was not hydrolyzed by trypsin or pepsin in the digestive tract. It was not attacked by putrefactive bacteria, and trypsin and pepsin were unable to hydrolyze it.

2.7.1.2 Toxicity of alkali-treated proteins

Toxic effects of severe alkali treatments had been observed by De Groot and Slump (1969) during rat feeding experiments. Diets containing relatively high levels of either soybean oil meal, casein or isolated soy protein treated with alkali (at pH 12.2, 40°C, 4 hr) were administered during the 4 to 6 week feeding regime. The results showed a slightly higher weight of liver and kidney with treated protein than in the control diet. Gain in body weight was not significantly affected in any of the experiments. Kidneys of female test rats showed distinct changes consisting of heavy calcereous deposits in the corticomedullary region, attended with distorted tubules. This phenomenon, nephrocalcinosis, is a common observation in the strain of rats used and occurs mainly in females. It is known to be aggravated in rats fed diets either low in calcium, high in phosphorus or low in magnesium. The results of the experiment showed that feeding rats with high levels of dietary protein revealed only an increased degree of nephrocalcinosis in females, which could be prevented by addition of calcium to the diet. Further, De Groot and Slump observed that feeding alkali-treated soy proteins to rats induced changes in kidney cells, characterized by enlargement of the nucleus and cytoplasm, increase in nucleoprotein, and disturbances in DNA synthesis and mitosis. The lesion, designated as nephrocytomegaly, affects the epithelial cells of the straight portion (*pars recta*) of the proximal renal tubules. Woodard and Short (1973) reported that renal cytomegaly of the *pars recta* is also induced by feeding rats synthetic lysinoalanine at a dietary level of 0.12%. Since this unusual, cross-linked amino acid is formed in proteins during alkaline treatment, the nephrotoxic action of the treated proteins was ascribed to the presence of lysinoalanine (Masters and Friedman, 1980).

The effect of dietary LAL on health is controversial. As mentioned above, Woodard and Short (1973) reported the occurrence of cytomegalia but no incidence of nephrocalcinosis, while De Groot and Slump (1969) found nephrocalcinosis but did not detect any sign of cytomegalia during the rat feeding experiments. In a later study, De Groot *et al.* (1973) again reported the occurrence of nephrocalcinosis in female rats (to a higher degree than in male rats) fed with alkali-treated, spun soy isolate, but indicated no occurrence of cytomegalia.

The conflicting reports on the toxicity of LAL from various researchers might be explained by amino acid racemization during alkaline processing of the test proteins. Some studies show that four different proteins subjected to the same alkaline treatment exhibited varying degrees of racemization (De Groot and Slump, 1969; De Groot *et al.*, 1973). It was suggested that the presence of D-amino acids would decrease enzymatic digestion of the proteins, thus restricting the amount of free LAL released. In order of cytotoxic effect, the most pronounced response is with free LAL, then low molecular weight LAL-containing peptides, followed by LAL-containing proteins (O'Donovan, 1976; Sternberg *et al.*, 1975). Thus, different proteins having the same LAL content will be expected to release differing quantities of free LAL, depending upon their extent of racemization.

De Groot *et al.* (1977) studied the effects of feeding free LAL to rats. The compound was fed as a dietary supplement at 0.1, 0.3 or 1.0% levels. Distinct growth depression and decreased food efficiency occurred in the high-dose group only. Upon examination of LAL at lower feeding levels, the only effect observed consisted of nephrocytomegalia at 100 ppm. The results indicate that the no-effect level of free LAL is between 30 and 100 ppm in the diet, or between 3.0 and 10.0 mg per kg body weight per day. Interperitoneal injections of free LAL (30 mg/day) for seven days resulted in renal lesions similar to those obtained by oral administration. Earlier, De Groot *et al*. (1976) found that protein-bound LAL had much less ability to induce cytomegalia than either free LAL or protein hydrolysates containing 1,000 ppm free LAL.

Karayianis (1976) reported that diets supplying 2,500 ppm LAL derived from alkali-heated lactalbumin did not induce the lesions, while 5,000 ppm LAL produced only mild cytomegaly. In contrast, 1,400-2,600 ppm LAL in treated soy protein produced extensive cytotoxicity. Since the lactalbumin was treated for 80 min at 60°C (Karayianis *et al*., 1979), little racemization (relative to soy protein at 60°C for 8 hr) would have occurred. The cytotoxicity of soy protein may be due to something other than, or in addition to, LAL content. It is possible that D-amino acid(s) may act synergistically with LAL in the expression of nephrocytomegaly. It has been reported that D-serine can induce renal lesions when fed to rats (Wachstein, 1947).

In an attempt to detect other factors which might be involved in the etiology of nephrocytomegalia (Van Beek *et al*., 1974), soy protein isolate produced for industrial nonfood uses and subjected to severe alkaline treatment, was fed to rats in a diet very similar in composition to that inducing renal lesions in the experiments of Woodard and Short (1973). The rats showed diarrhea and gained weight at an abnormally low rate, but did not reveal renal cytomegalia at the termination of the experiments after 8 weeks. The poor nutritional quality of the diet was found to be caused mainly by deficiency of threonine and methionine. Though no evidence was obtained for these deficiencies to be of significance for the induction of renal cytomegalia, it was stressed that the use of a nutritionally well-balanced diet is a prerequisite to prevent complications in interpreting the results of toxicological experiments.

Although studies showed that clinical test animals are susceptible to nehprocytomegaly induced by LAL, this biological effect is not observed in humans. Sternberg and Kim (1977) found that LAL existed in a number of home-cooked and commercial foods which had not been alkali-treated. For example, the egg white of egg boiled three minutes contained 140 ppm of LAL, while dried egg white powder contained from 160 to 1,820 ppm of LAL, depending on the manufacturer. No LAL was found in fresh egg white. Sternberg and Kim noted that β-elimination and addition of lysine to the double bond of dehydroalanine reduce the level of the essential amino acid lysine. They suggested that this can be prevented by other amino acids, such as cysteine, added to the reaction. Recently, Satterlee and Chang (1982) concluded that the greatest problem associated with LAL would be the lowered nutritional quality of proteins which contain high levels of LAL, especially if these proteins were the major source of protein in the diet. From the processing standpoint, when contact between food and alkali is kept to a minimum at the lowest temperature possible with adequate control of mixing, there is no apparent reason to discontinue the use of alkali.

2.7.2 Nutritional quality of texturized cheese whey proteins

Another raw material that might be suitable for protein texturization is whey, which is considered as a highly nutritious by-product of cheese and casein manufacture, containing about 0.6% protein. The whey proteins represent the non-casein proteins as well as the fractions and fragments of casein which remain soluble when the caseins have been precipitated enzymatically by rennet or isoelectrically by acid.

The high nutritive value of whey proteins is mainly due to their high content of essential amino acids (Forsum and Haembraeus, 1974). It has been reported that whey protein concentrate has a surplus of all essential amino acids compared with the FAO pattern (Forsum, 1975). Of special interest are the high contents of isoleucine, lysine, threonine and tryptophan.

Lohrey and Towler (1979) noticed significant differences in the rehabilitation of rats which had been fed with low protein (3%) diet. When fed a diet containing 12% protein, weight gains for the first week were much the same for casein and whey protein. Weight gains for the second week were significantly greater with whey protein. Lohrey and Towler noticed quite the same effect in rats fed on a standard diet of 10% protein. Initially, weight gains were similar, but in later weeks rats being fed on whey proteins showed significantly higher weight gains indicative of the effects of some limiting amino acids which became apparent at later stages. In earlier experiments, Lohrey *et al.* (1978) showed that oxidized lipid reacted with the proteins, resulting in loss of availability of certain amino acids (e.g. methionine) and a reduction in apparent digestibility.

Owing to the limited resources on the use of whey proteins in texturization processes, information leading to any nutritional impairment as a result of the process remains nil.

2.7.3 Need for nutritional assessment of texturized protein foods

The nutritional quality of any processed food is significantly related to the nature of the processing conditions. However, in-depth knowledge of the events occurring in the food system under varying conditions provides a means for adequate control of the process. For instance, in protein texturization a clear understanding of the mechanism involved in the texturization should help define the conditions necessary to bring about the desired textural effect. This means that conditions causing detrimental nutritional effects (e.g. LAL production in an alkaline environment) may be minimized or eliminated, while still meeting the required physicochemical and sensory qualities in the final product. Further, a greater understanding of the events leading to protein texturization should lead to the maximum utilization of proteins from various conventional and non-conventional sources. It would be advantageous, therefore, to produce a textured protein material which not only has a highly defined fiber structure but also has a highly supplemented amino acid content. This means that a protein source deficient in a particular amino acid can be texturized with another protein source having the limiting amino acid. Such a case would be beneficial to developing countries wherein the price of meat is excessively expensive and protein deficiency has been a plaguing nutritional problem.

3. MATERIALS AND METHODS

3.1 Characterization of the Alkali-Extracted Proteins from Mechanically Deboned Poultry Meat Residues

3.1.1 Sample preparation

Protein isolate. Mechanically deboned poultry meat residues were obtained from a local poultry processing plant. The bone residues were collected during the deboning operation and immediately extracted following the basic procedure described by Lawrence and Jelen (1982).

Preliminary experiments showed that a considerable amount of fat was recovered when 20 kg batches of deboning residues were extracted. This problem was minimized following a minor modification in the process (Figure 3.1). Extraction was done using a tank (Univat, Cherry-Burrell Corp., Chicago, IL, USA), operated with a mechanical mixer. The solids were separated from the alkali extract using a cheese press (Damrow Brothers Co., Fond Du Lac, WI, USA) with the basket with three layers of cheese cloth. The liquid extract was kept at 5°C for about 15 min to allow separation of the fat and then skimmed off from the top layer. The alkali extract was clarified by centrifugation at 27,300 x g using a Sorvall centrifuge (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Inst.) with rotor GSA, GS-3, for 30 minutes at 1 to 2°C; allowing further fat separation. The solubilized proteins in the clear alkali

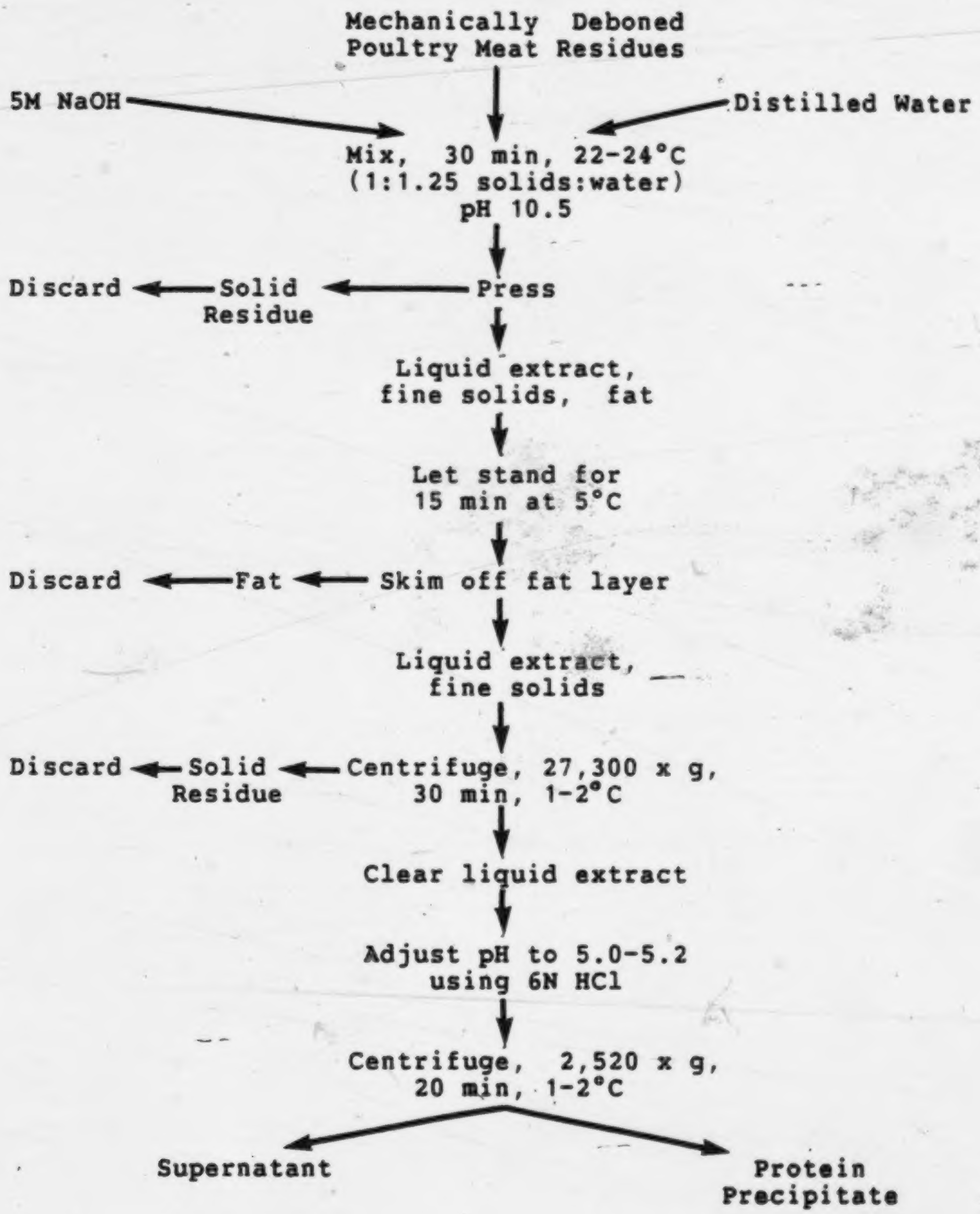

Mechanically Deboned Poultry Meat Residues
5M NaOH
Distilled Water
Mix, 30 min, 22-24°C (1:1.25 solids:water) pH 10.5
Discard
Solid Residue
Press
Liquid extract, fine solids, fat
Let stand for 15 min at 5°C
Discard
Fat
Skim off fat layer
Liquid extract, fine solids
Discard
Solid Residue
Centrifuge, 27,300 x g, 30 min, 1-2°C
Clear liquid extract
Adjust pH to 5.0-5.2 using 6N HCl
Centrifuge, 2,520 x g, 20 min, 1-2°C
Supernatant
Protein Precipitate

Figure 3.1 Aqueous extraction of protein from mechanically deboned poultry meat residues.

extract were separated by isoelectric precipitation (pH 5.0-5.2), using 6N HCl as the precipitant. Continuous agitation was provided in order to prevent lump formation of the precipitated protein. The isolated protein was immediately used in the freeze texturization experiments.

Mechanically deboned poultry meat paste (MDPM). Industrially produced MDPM was obtained from POS Pilot Plant Corp. in Saskatoon. The protein from this paste was also extracted following the procedure present in Figure 3.1, excluding the pressing step.

3.1.2 Proximate analyses of protein extract

Protein content. The protein contents of all samples were determined using the micro-Kjeldahl method (AOAC, 1975).

Fat content. The fat contents of the protein extracts were determined by the Soxhlet method (AOAC, 1975).

Moisture content. The moisture content of the protein extract was determined by drying about 5 g sample in a vacuum oven at 80°C for 24 hr.

Ash content. Ash determination was carried out at 500°C using a muffle furnace.

3.1.3 Hydroxyproline content

The proportion of collagen in the protein isolate was determined by hydroxyproline analysis, using Method 1 as described by Woessner (1961).

A sample containing 50 mg protein was hydrolyzed in 1.0 ml 6N HCl at 130°C for 3 hr. After neutralizing the hydrolysate with 2.5N NaOH, 2 ml of hydrolysate were collected and mixed with 1 ml of 0.05M Chloramine T (sodium *p*-toluene sulfonchloramide) and then allowed to stand for 20 min at room temperature. Then 1 ml of 3.5M perchloric acid was added into the solution, mixed and allowed to stand for 5 min, followed by addition of *p*-dimethylaminobenzaldehyde solution. The resulting solution was incubated in a 60°C water bath for 20 min and then cooled in tap water for 5 min. The absorbance of the solution was determined spectrophotometrically at 557 nm using a Beckman DU-8 UV-Visible spectrophotometer (Beckman Instr., Inc., Irvine, CA, USA). The hydroxyproline values were determined directly from the standard curve (see Appendix).

3.1.4 Total sulfhydryl group content

The total amount of sulfhydryls in the protein extract was determined using the method described by Sedlak and Lindsay (1968). Five grams of protein was homogenized in 50 ml of 0.02M EDTA using a Sorvall Omni Homogenizer (Ivan Sorvall, Inc., Newton, CT, USA), in an ice bath for 2 minutes.

Aliquots of 0.5 ml of the protein homogenates were mixed with 1.5 ml of 0.2M tris buffer, pH 8.2, and 0.1 ml of 0.01M DTNB (5,5'-dithiobis-(2-nitrobenzoic acid). The mixture was brought to 10.0 ml with 7.9 ml of absolute methanol, allowed to stand for 30 minutes, then filtered twice through a Whatman no. 42 filter paper. The absorbance of the clear filtrate was read in a Beckman DU-8 UV-visible spectrophotometer at 412 nm. The concentration of the cysteine in the protein sample was determined directly from the standard curve (see Appendix).

3.1.5 Calcium, magnesium and sodium contents

These elements were determined by atomic absorption spectrophotometry following the procedure described by Wallace and Satterlee (1977).

About two grams protein sample was ashed in a muffle furnace at 550°C for 12 hours. The ash was dissolved in 6N HCl to a total volume of 50 ml. Proper dilutions were prepared using 1% La as diluent for the analyses of Ca and Mg, while 1.5 mM LiCl was used for Na. The absorbances of the test and standard solutions were determined using an atomic absorption spectrophotometer (Pye Unicam SP 2900 Atomic Absorption (Double Beam) spectrophotometer), with wavelengths of 423, 285 and 589 nm for Ca, Mg and Na, respectively. The amount of each of these elements was determined directly from the prepared standard curves, as shown in the Appendix.

3.1.6 Purification of the protein isolate

The method described by Parson and Lawrie (1972) was employed in the purification of the protein sample for molecular weight determination. The protein isolate was homogenized in three volumes of 0.1N HCl in 75% ethyl alcohol. The homogenate was centrifuged at 30,000 x g for 30 min, the supernatant was discarded, and the pellet was resuspended in five volumes of acetone. The suspension was left 3 hr at room temperature and centrifuged again. The acetone extraction was done twice and after the final centrifugation the pellet was dried at 50°C and then finely ground. The protein content of the dried protein powder was about 95%, estimated by the micro-Kjeldahl method.

3.1.7 Preparation of sample for electrophoresis

The protein powder was dissolved in 1 ml of sample buffer which was composed of 0.05 M tris-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue and 30% glycerol. Twenty μl of 2-mercaptoethanol per ml protein sample was also added. The mixture was heated in a boiling water bath for about 2 min prior to electrophoresis.

3.1.8 Vertical slab electrophoresis technique

The protein extract was resolved in acrylamide gels using a vertical slab electrophoretic technique which has been developed at the Department of Food Science, University of Alberta, based on the design described by Akroyd (1967) and Laemmli (1970). The Vertical Slab Electrophoresis Cell Model 220 from Bio-Rad Labs. (Canada) was used in this study.

3.1.9 SDS-polyacrylamide gel electrophoresis (SDS-Page)

The chemicals used in SDS-PAGE were purchased from Sigma Chem. Co. (St. Louis, MO, USA). The running gels were prepared by using a 7.5%-20% gradient gels. The formulations of the stock and working solutions are listed in Table 3.1.

The gels were cast between two glass plates (17.8 cm length x 14.0 cm width) and spaced 1.5 mm apart. The gradient gel was prepared by mixing 7.5% and 20% gel solutions, placed separately in mixing chambers. After polymerization (about 3 hr), the sample well-forming comb was inserted between the glass plates. Using a Pasteur pipet, the stacking gel was added at the end and between wells to overflowing. After polymerization, the comb was removed as was the excess liquid in the sample wells.

A discontinuous buffer system was employed: upper and lower vessel buffer, both of which were filled with the running buffer. Standard proteins of approximately 5-10 mg/ml dissolved in the sample buffer, were underlayered into the bottom of the wells using a microliter syringe. The standard proteins (Pharmacia Fine Chemicals, Uppsala, Sweden) used in this study are given in Table 3.2.

Protein Sample: Approximately 150 μg were applied in each sample well. This predetermined amount of protein gave sharp protein bands. Electropheresis was carried out at 30 mA for 3-4 hr or until the tracking gel reached the bottom of the gel. After the electrophoretic run, the gel was removed from the plates and then stained in a solution of

Table 3.1 SDS-Page slab gel and buffer system formulations.

	Composition	
I. Stock Solutions		
Lower buffer stock	1.5 M Tris-HCl, pH 8.8	
Upper buffer stock	0.5M Tris-HCl, pH 6.8, 0.4% SDS	
Running buffer stock	0.3M Tris, 0.19M glycine 0.1% SDS (per liter, diluted 10x before use)	
Acrylamide stock	30% acrylamine, 0.8% N,N'-methylene-bis-acrylamide	
Ammonium persulfate	3.3% $(NH_4)_2S_2O_8$	
II. Running Gel		
	7.5%	20%
Lower buffer stock, ml	3.00	3.00
Acrylamide stock, ml	3.00	8.00
Glycerol (60%), ml	0.54	1.05
Water, ml	5.40	--
3.3% $(NH_4)_2S_2O_8$, ml	0.06	0.06
N,N,N',N'-tetramethylene-diamine (TEMED), μl	5.00	5.00
III. Stacking Gel		
Upper buffer stock, ml	2.5	
Acrylamide stock, ml	1.5	
Water, ml	6.0	
3.3% $(NH_4)_2S_2O_8$, μl	91.0	

Table 3.2 Protein standards used as markers in SDS-Page.

Protein Standard	Molecular Weight
Carbonic Anhydase	29,000
Albumin, Egg	45,000
Albumin, Bovine	66,000
Phosphorylase B	97,400
B-Galactosidase	116,000
Myosin	205,000

45% methanol, 10% acetic acid and 0.35% Coomasie Blue R-250.

The background stain was removed by immersing the gels in a destaining solution of 10% acetic acid and 30% methanol. The gels were stored in a solution of 7.5% acetic acid and 5% methanol. The mobilities of each protein subunit were calculated and the molecular weights estimated from the standard curve (Figure 3.2), obtained by plotting the log molecular weight versus mobilities of the standard proteins.

3.1.10 Determination of amino acid composition

The purified protein sample was submitted to the College of Dentistry, University of Alberta, for amino acid analysis. The sample was hydrolyzed in 5.7M HCl, in the presence of 5% (w/v) phenol, at 110°C for 22 hr. The analysis of the amino acids was done using a Beckman Amino Acid Analyzer Model 121 MB.

3.2 Freeze Texturization Studies

3.2.1 Freezing technique

About 150 g of the fresh protein isolate (9% protein by micro-Kjeldahl method) was frozen in an aluminum dish (7.0 cm x 3.3 cm) fitted at the center of a styrofoam block (17cm x 17cm x 10cm), to insulate all sides except the top portion of the dish (Figure 3.3). This freezing method was based on that reported by Lugay and Kim (1981) and was used to achieve unidirectional freezing of the protein mass. The

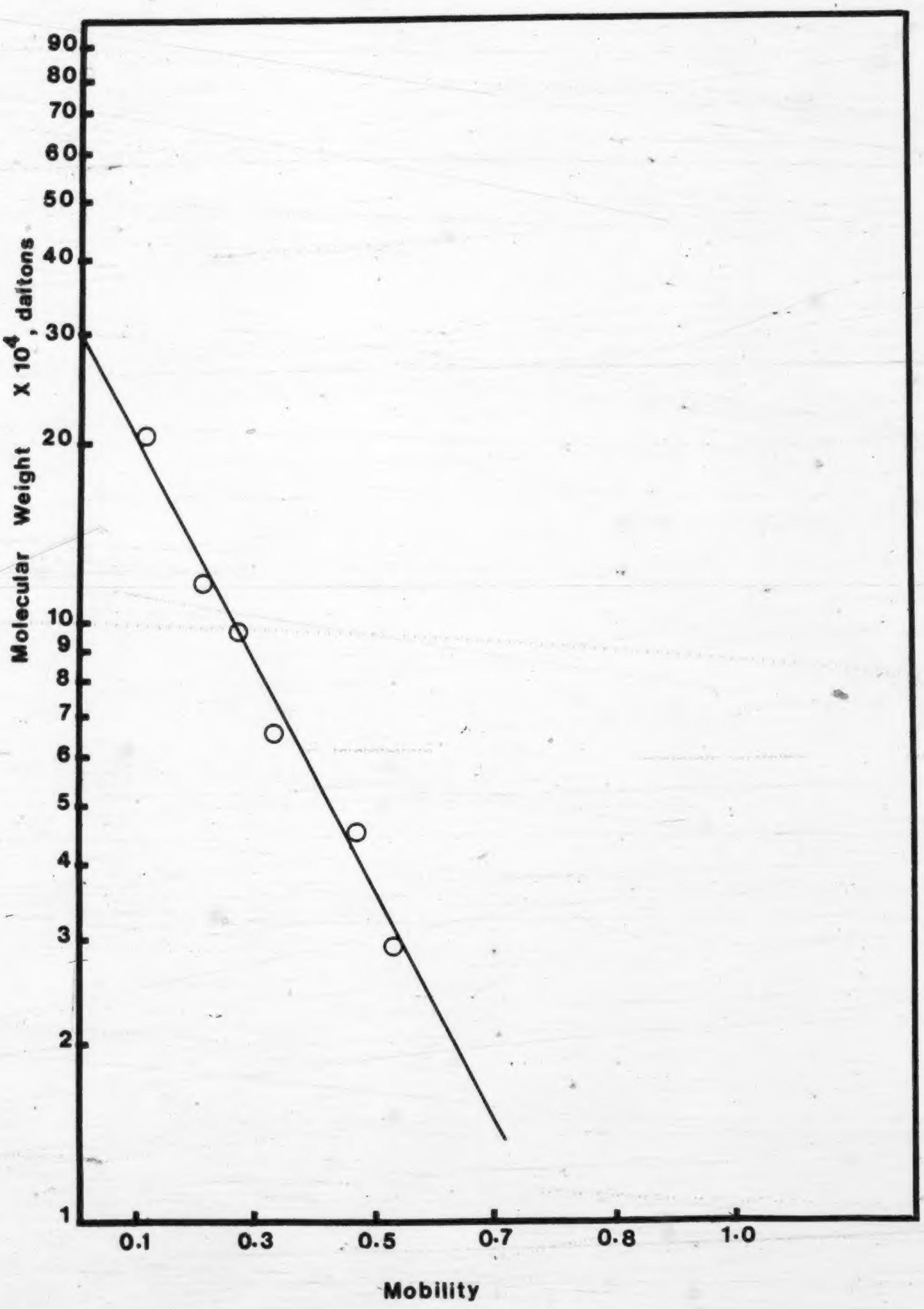


Figure 3.2 Standard protein calibration curve. Protein standards are listed in Table 3.2.

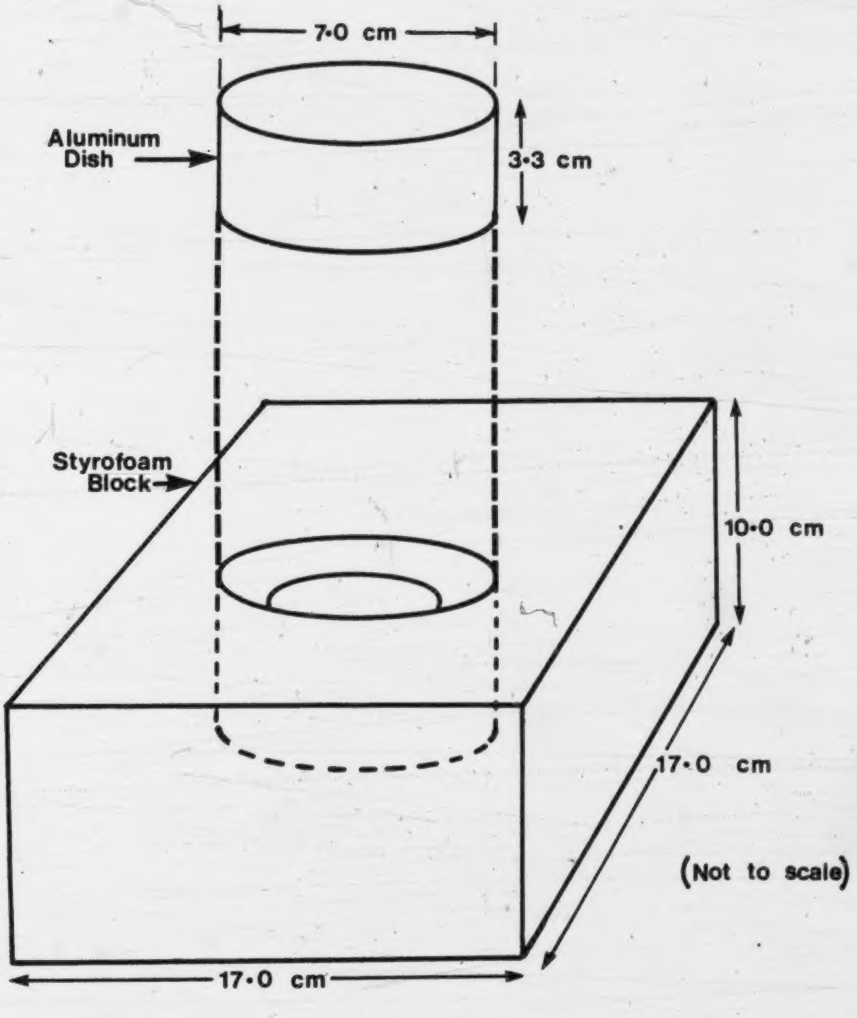

7·0 cm
Aluminum Dish
3·3 cm
Styrofoam Block
10·0 cm
17·0 cm
(Not to scale)
17·0 cm

Figure 3.3 Schematic representation of the freezing mould used in the unidirectional freezing technique.

samples were frozen by placing in a freezer at -25°C or -60°C and held at this temperature for 48 hours. Following freezing of the protein slurry, the insulation mould was removed and frozen proteinaceous material allowed to thaw at room temperature. The freeze texturized protein was heat set by autoclaving at 121°C, 1.034 bar, for 15 min, and then cooled at room temperature. These pre-determined heat-setting conditions irreversibly fixed the parallel oriented protein sheet structures.

3.2.2 Effect of alkali and acid treatments on fiber formation

Different sample preparations were used in order to study fiber formation and cross-linking in the alkali-extracted, acid-precipitated protein. Preliminary experiments had indicated the difficulty of visualizing the protein fibers in the crude protein isolate. However, washing the unfrozen protein isolate with 500 ppm of EDTA, followed by washing with deionized water, removed a considerable amount of "extraneous" materials that could have masked the protein fibers.

Fiber formation was observed in both the unfrozen and freeze texturized protein isolates. The fresh protein isolate was dehydrated by successive immersion in 75, 85, and 95% ethyl alcohol (15 min in each), which also provided stabilization of the protein. On the other hand, some freeze texturized samples were freeze dried in order to fix the structure and this technique was employed to correct any possible alteration that might have been caused by stabilizing the protein in ethyl alcohol.

3.2.3 Effect of freezing rate on fiber formation

Protein isolates were frozen at -25 and -60°C using a walk-in freezer and at -196°C with liquid nitrogen. The frozen samples were thawed in serial concentrations of ethyl alcohol for dehydration and stabilization.

3.2.4 Texturization of mechanically deboned meat paste

The behaviour of poultry meat paste and of the protein extracted by alkali from this paste after freeze texturization was compared by unidirectionally freezing them at -25°C. Both samples were thawed at room temperature and the characteristic protein sheet structure formation was observed visually.

3.2.5 Effect of pH, calcium, urea and freezing temperature on texturization

The pH of the protein isolate was adjusted from 5 to 7 or 9 using 5M NaOH. The alkali was added dropwise with continuous mixing in order to prevent localized gel formation. Each pH adjusted sample was treated, separately, with different concentrations of calcium (0; 2.50; 6.20; 12.5; and 18.8, x 10^{-2} moles/l) added as $CaCl_2$, or urea (0; 0.10; 0.40 and 0.70 moles/l). The final volume of the samples under each pH and chemical treatments was maintained constant by adjusting it with distilled water. The prepared samples were placed in the freezing mould (Figure 3.3) and then frozen at -25 or -60°C for 48 hr. After freezing, the samples were thawed at room temperature and then heat set by autoclaving at 121°C, 1.034 bar for 15 min. The expressed liquid from the thawed samples was collected to determine the amount of protein that remained soluble following the various treatments.

3.3 Determination of Soluble Protein after Texturization

3.3.1 Expressed liquid

The amount of protein in the expressed liquid, the liquid that separated from the freeze texturized protein mass upon thawing at room temperature, was determined following the Lowry method.

Five milliliters of reagents A and B (A--2% Na_2CO_3 in 0.1N NaOH; B--0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium citrate, mixed at a ratio of 50:1, A:B) were added into 1 ml of the expressed liquid. The mixture was allowed to stand for 10 min at room temperature and then 0.5 ml of 1N Folin-Ciocalteau reagent was added. After mixing, the resulting solution was allowed to stand for 30 min and the absorbance determined at 750 nm using a spectrophotometer. The protein content in the sample was extrapolated from the standard curve using bovine serum albumin (Sigma Chem. Co., St. Louis, MO, USA) as the standard protein (Appendix 6). The determinations were made in triplicate.

3.3.2 Protein isolate

The nature of chemical bonds present in the protein isolate, following the three main stages of freeze texturization, was determined through the solubility of the protein in various solvents. The solutions used were water, 0.6M KCl, 1.5M urea, 8M urea, 1M KOH and 1% SDS. About 0.5 g samples of the freshly prepared protein isolate, thawed freeze texturized protein and heat-set, freeze-texturized protein, were homogenized in 5 ml of each of the test solvents. The samples were continuously agitated for 1 hr using a mechanical shaker.

The undissolved protein was then separated by centrifugation at 15,000 rpm for 30 minutes at 1-2°C (Sorvall GS-34 rotor). The protein content of the clear supernatant was determined by using the Lowry method as described above. The determinations were made in triplicate.

3.4 Measurement of Textural Integrity

The method used in the measurement of textural integrity was patterned from that of Cegla *et al.* (1978). The thawed freeze-texturized protein samples contained in aluminum moisture dishes, were heat set in an autoclave for 15 min at 1.034 bar and 121°C. The samples were taken out from the autoclave and cooled at ambient temperature prior to texture measurement.

About 5 mm thickness from the surface of the texturized protein was trimmed off before sampling for texture measurement. Ten 15-g samples (from each replicate) were cut into 0.5 cm^3 pieces and tested with a 9-wire (20 cm^2 cross-sectional area) Ottawa Texture Measuring System (OTMS) food cell connected to a Type TM 1130 Instron Testing Machine (Instron Eng. Corp., Canton, MA, USA). A crosshead speed of 50 mm/min and chart speed of 200 mm/min gave well defined force-deformation curves. The compression head was stopped 1.5 mm from the wire grid. From the force-deformation curve, the compressive strength was determined as the maximum force required to compress and extrude the freeze-texturized protein samples through the wire grid.

3.5 Scanning Electron Microscopy

Freeze texturized protein was sliced into 0.5 cm^3 pieces using a sharp razor blade. The technique for scanning electron microscopy was based on that suggested by the Department of Entomology, University of Alberta. The sliced protein samples were dried using the critical point drying technique, with liquid CO_2. The samples were mounted on aluminum stubs coated with silver conducting paint and sputter coated with gold to a thickness of 150 A. Scanning electron microscopic examination was performed with a Cambridge Stereoscan 250 at 25 Kv.

3.6 Macrophotography

Cylindrical samples of 2.3 cm dia x 0.5 cm thick were cut from the product cores using a cork borer (No. 15). This represents the surface perpendicular to the direction of heat flow during the unidirectional freezing. Sliced samples (2.3 cm x 2.0 cm x 0.5 cm) were also prepared to show the surface parallel to the direction of heat flow. All samples were submitted to Photographic Services, University of Alberta, for photography.

3.7 Freeze Texturization of Whey Protein Concentrate

3.7.1 Recovery of whey proteins

Cottage cheese whey (pH 5.4) was collected immediately after cheese processing, from a local cheese manufacturing plant. The whey was allowed to stand for about 12 hr at 5°C, to permit sedimentation of the fine cheese particles. The clear whey was siphoned off, and the soluble whey proteins were recovered by ultrafiltration, acid-heat precipitation and sodium hexametaphosphate precipitation methods according to the procedures shown in Figures 3.4, 3.5 and 3.6, respectively.

3.7.2 Alkali-solubilization of whey proteins

The effect of alkali-solubilization of whey proteins on the freeze texturization behaviour of these proteins was studied. The freeze dried, acid-heat precipitated whey

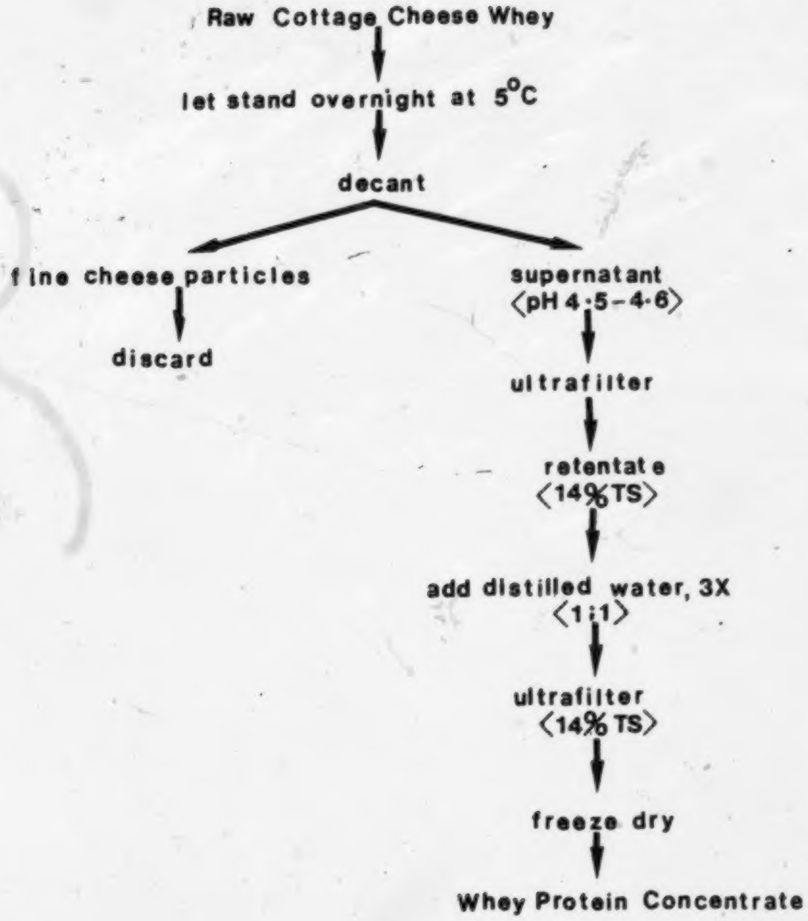

Raw Cottage Cheese Whey
let stand overnight at 5°C
decant
fine cheese particles
discard
supernatant
<pH 4.5-4.6>
ultrafilter
retentate
<14% TS>
add distilled water, 3X
<1:1>
ultrafilter
<14% TS>
freeze dry
Whey Protein Concentrate

Figure 3.4 Concentration of proteins from cottage cheese whey by ultrafiltration.

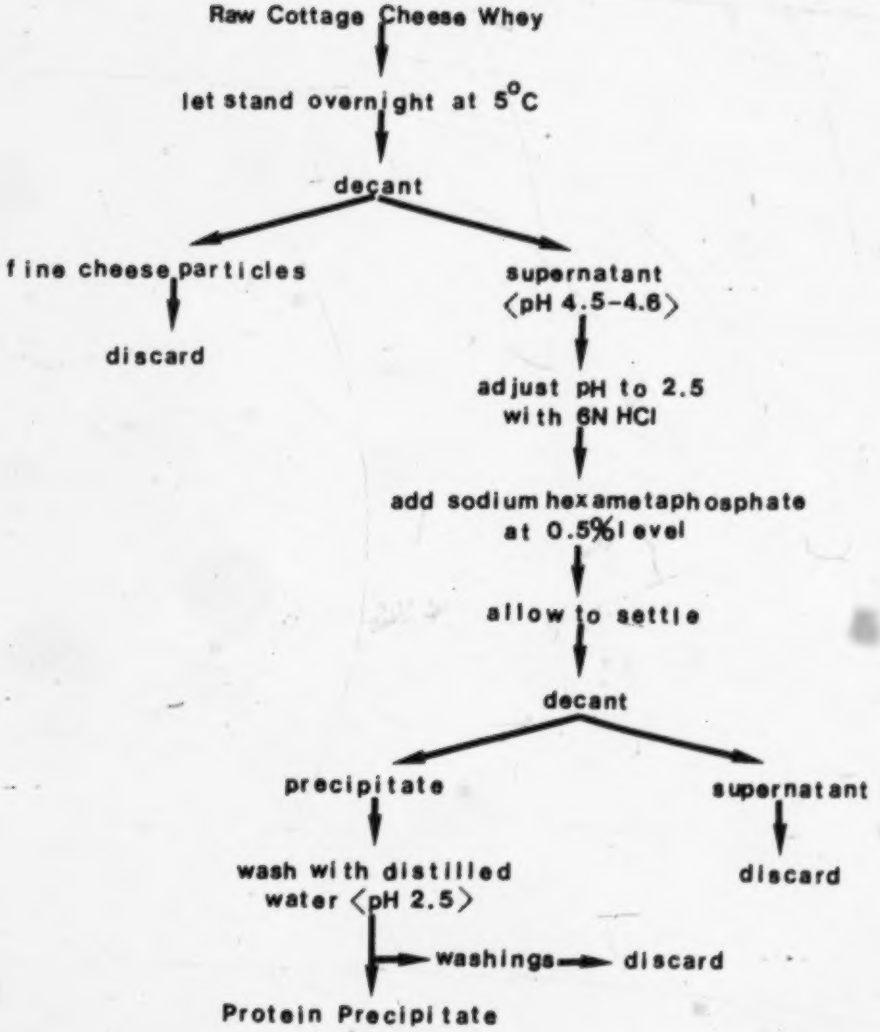

Raw Cottage Cheese Whey
let stand overnight at 5°C
decant
fine cheese particles
discard
supernatant
<pH 4.5-4.6>
adjust pH to 2.5
with 6N HCl
add sodium hexametaphosphate
at 0.5% level
allow to settle
decant
precipitate
supernatant
discard
wash with distilled
water <pH 2.5>
washings
discard
Protein Precipitate

Figure 3.5 Precipitation of proteins from cottage cheese whey with polyphosphates (Hartman and Swanson, 1966).

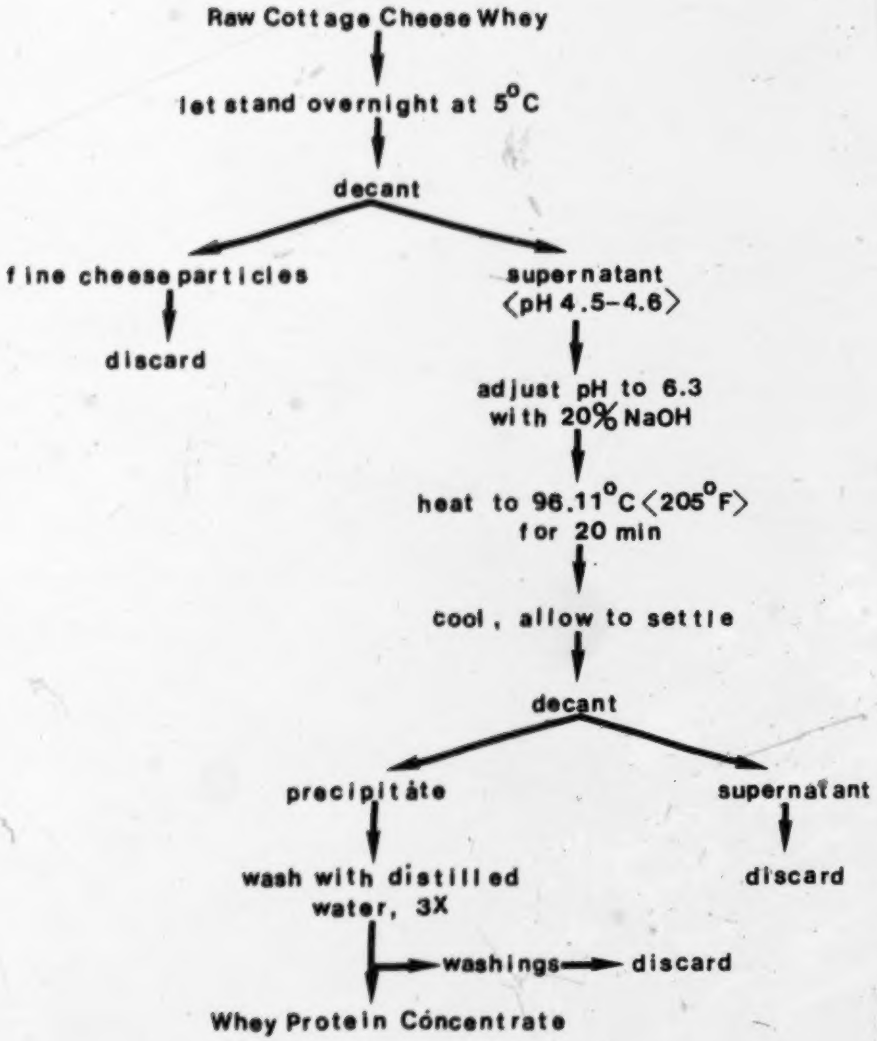

Raw Cottage Cheese Whey
let stand overnight at 5°C
decant
fine cheese particles
discard
supernatant
<pH 4.5-4.6>
adjust pH to 6.3
with 20% NaOH
heat to 96.11°C <205°F>
for 20 min
cool, allow to settle
decant
precipitate
wash with distilled
water, 3X
washings
discard
Whey Protein Concentrate
supernatant
discard

Figure 3.6 Acid-heat precipitation of proteins from cottage cheese whey.

protein (pH 5.6) was dispersed in distilled water using a mechanical stirrer. The pH was adjusted to 11.2 with 5N NaOH while the protein dispersion was continuously agitated. The protein solution was heated at 75°C for 2 min (Jelen and Schmidt, 1976). After cooling, the pH of the protein solution was readjusted to 5, followed by centrifugation at 2,520 x g for 20 min at 1-2°C.

3.7.3 Freeze texturization

The same technique as used with poultry meat protein was followed in the freeze texturization of the whey protein concentrate.

4. RESULTS AND DISCUSSION

4.1 Characterization of the Alkali-Extracted, Acid-Precipitated Poultry Meat Residue Protein

4.1.1 Proximate composition

The chemical composition of the alkali-extracted, acid-precipitated protein obtained from mechanically deboned poultry meat residues is shown in Table 4.1. Separation of the protein precipitate from the supernatant by centrifugation at 2,520 x g gave a protein isolate containing ca. 90% moisture or ca. 10% total solids. Preliminary experiments had indicated that this moisture content provided sufficient ice crystal formation to effect freeze texturization of the ca. 9% protein present in the isolate. This was noted in the ability of the texturized protein to maintain its form following thawing at room temperature and the heat setting process. The presence of the parallel-oriented protein sheet structures could be easily visualized in the final product. Although the protein could be texturized at lower solids content (<10%), the structure collapsed upon thawing and suffered considerable shrinkage following the heat setting conditions used in this study. This made it difficult to ascertain the formation of the texturized structure due to the freezing process. On the other hand, higher total solids content (e.g. 20%) demonstrated textured structure, however, the parallel structures were too compact to permit

Table 4.1 Chemical composition of alkali-extracted, acid-precipitated protein obtained from mechanically deboned poultry meat residues.

Composition	Amount'
Moisture, %	88.91 ± 0.70
Crude Protein, %	8.84 ± 0.95
Crude Fat, %	2.54 ± 0.08
Total Ash, %	0.36 ± 0.04
Total Sulfhydryls, mmoles/g	4.84 ± 0.77
Hydroxyproline, mg/g	1.47 ± 0.31
Sodium, ppm	126.38 ± 7.51
Calcium, ppm	90.44 ± 5.55
Magnesium, ppm	3.50 ± 0.67

' ± indicates standard deviation.

visualization of the mechanical compression effect of ice upon the protein mass.

Kim and Lugay (1978a,b) noted that the quality of the fibers produced at low solids content is high. They also stated that, generally, the optimum concentration for freezing will be anywhere from 3% to about 35% protein, with concentrations of 10-30% being preferred, based upon the total weight of the aqueous protein mixture.

The proximate composition of the protein isolate used as a texturizing material was similar to that employed by Lawrence (1981). The fat content of the isolate (ca. 2.5%) was also within the level used by other workers. Successful texturization of soy, beef or poultry meat proteins was obtained in the presence of 2.5 to 10% fat (w/w) in the protein slurries (Middendorf *et al.*, 1975). The alkali extraction conditions used by Lawrence (1981) were strictly followed in the present study. Thus, in accordance with his results, it was assumed that the formation of lysinoalanine during the extraction process was negligible.

4.1.2 Hydroxyproline content

Since the protein recovered from the deboning residues has a high potential as a food ingredient, it is important to know if other muscle protein components of less significant usage are also included in the alkaline extract. The presence of collagen, one of the connective tissue proteins, is considered undesirable both from the standpoint of protein functionality and nutrition. Some of the problems associated with collagen are: 1) decreased emulsifying capacity of meat because of its poor solubility; 2) lower water holding capacity of meat since collagen has a low content of charged and hydrophilic amino acids; and 3) low nutritional value because of the low proportion of the nutritionally essential amino acids (Goll et al, 1977).

Hydroxyproline has been widely used as an indicator of the presence of collagen since it is thought to be confined almost exclusively to the connective tissue scleroprotein (Woessner, 1961). The low hydroxyproline content (1.47 mg/g of fresh sample or 1.67 g/100 g dry protein) in the protein isolate obtained in this study (Table 4.1) indicated that the alkaline conditions employed during protein extraction do not cause significant solubilization of collagen present in the deboning residues (Golan and Jelen, 1979; Lawrence, 1981). Similar values were obtained by Swingler and Lawrie (1979) during protein extraction at pH 10.0 and precipitation at pH 5.0. Proteins isolated from bovine lung and rumen contained 2.3 and 1.2 mg/g, respectively, after 2 hr extraction at 20°C.

4.1.3 Minerals

The low calcium content of the protein extract (Table 4.1) is a clear indication of the low amount of fine bone particles present in the ground deboning residues. Watt and Merrill (1963) stated that, since the calcium content of meat is very low (0.01%) and the amount is relatively constant, any calcium increase in mechanically deboned meat over that found in fat and lean is an indication of increased bone particles. Under the USDA's proposed rules, mechanically deboned meat can have a maximum of 0.75% calcium and that for processing can have a maximum of 1.0% calcium (Field, 1976).

The high amount of sodium in the protein isolate compared to calcium or magnesium is understandable since the extraction was carried out at pH 10.5, which requires a considerable amount of sodium hydroxide, in adjusting the pH to the isoelectric point of the major meat proteins (myosin and actin), in addition to that actually present in the deboning residues. From the nutritional standpoint, that is, considering the recent controversial issues on the effects of excessive amounts of sodium in the human body (i.e., hypertension), the sodium content of the protein isolate is negligible compared to the other protein isolates or concentrates commonly used in food processing (Table 4.2).

4.1.4 Total sulfhydryl groups

The total sulfhydryl group content in the protein samples was determined as cysteine. The bulk of the sulfhydryl groups in muscle is bound to proteins. For example, myosin has 40 sulfhydryl groups, while actin has 5-6 groups (Cheftel, 1958; Hofmann and Hamm, 1978).

Table 4.2 Sodium content of protein sources used in food product formulations.

Protein Source	Amount of sodium, ppm
Mechanically deboned broiler[1] backs, and neck, with skin	400
Non-fat dry milk[1]	5,350
Soy protein[1]	10,000
Calcium-reduced skim milk[1]	22,800
Sodium-caseinate whey blends[1]	13,600
Alkali-extracted protein[2]	130

[1] From Marsh (1983).

[2] From Table 4.1 (this work).

Since centrifugation does not completely separate the precipitated myofibrillar proteins from the soluble sarcoplasmic proteins, it can be assumed that a small proportion of the latter group of proteins is also present in the protein extract. In the present study, centrifugation at 2,520 x g resulted in a protein isolate with approximately 90% moisture, hence, a considerable amount of soluble sacroplasmic proteins. This should also explain the presence of numerous protein bands in the SDS-PAGE electrophoretogram, as will be discussed later. Several SH groups are present in the sarcoplasmic reticulum. For example, the lipoproteins contain 24 half cystine equivalents per 10^5 gram protein and the 54,000 dalton protein, $7.4/10^5$ gram (detected as cysteic acid) (Maclennan *et al.*, 1973). The presence of sulfhydryl groups in the protein isolate may lead to intra- and intermolecular cross-linking during the protein texturization processes through disulfide-sulfhydryl interchange reactions (Hashizume *et al.*, 1971; Fukushima, 1980; Kelley and Pressey, 1966).

4.1.5 Molecular weight distribution

The mechanically deboned poultry meat residue is composed of meat, skin, bones, blood and connective tissues. Therefore, it is expected that the protein extracted from this source would yield numerous protein subunits owing to its heterogeneity. In the present study, the purified protein isolate was resolved on SDS-PAGE and the molecular sizes of its subunits determined by comparison with marker proteins. The electrophoretograms of the alkali-extracted, acid-precipitated protein and the standard proteins are shown in Figure 4.1. The numerous bands indicate a wide range of protein subunits present in the isolate. Quite evident is the presence of the dark bands, which indicates high protein concentrations, two of which should correspond to myosin and actin (with molecular weights of 220,000 and 46,000-47,000 daltons, respectively; Hoffmann and Hamm, 1978). The molecular weights of the protein subunits resolved using the SDS-PAGE technique are presented in Table 4.3.

It has been established that the texture of meat products is inherently dependent upon the fibrous nature of the meat. Likewise, the presence of a fibrous structure is an important factor in fabricated meat-like products (Kim and Lugay, 1978a,b). Thus, the presence of high molecular weight myofibrillar proteins in the protein isolate makes this raw material highly suitable for texturization purposes. Quite important is the fibrous or extended nature of these meat proteins (Goll *et al.*, 1977) required for maximum protein-protein interactions to effect texturization (Schmidt, 1981; Wall and Huebner, 1981).

In a study using a salt-extraction method of protein recovery from poultry deboning residues, Kijowski and Niewiarowicz (1985) suggested that the presence of numerous protein bands in the electrophoretogram was due to the

Table 4.3 Molecular weight distribution of protein subunits in alkali-extracted, acid-precipitated protein obtained from poultry deboning residues.

Band Number[1]	Approximate Molecular Weight, daltons[2] (± SD)
1	247,500 ± 1,000
2	233,300 ± 1,000
3	223,300 ± 1,000
4	201,700 ± 3,000
5	155,500 ± 3,000
6	140,500 ± 3,000
7	134,200 ± 4,000
8	126,700 ± 5,000
9	110,000 ± 4,000
10	93,800 ± 3,000
11	79,800 ± 4,000
12	73,000 ± 1,000
13	65,600 ± 3,000
14	58,300 ± 1,000
15	54,800 ± 2,000
16	50,700 ± 3,000
17	52,500 ± 3,000
18	45,300 ± 1,000
19	42,000 ± 3,000
20	35,700 ± 2,000
21	26,800 ± 840
22	21,500 ± 100
23	18,750 ± 1,000
24	18,500 ± 800

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[1] See Figure 4.1.

[2] Average values of three replicates.

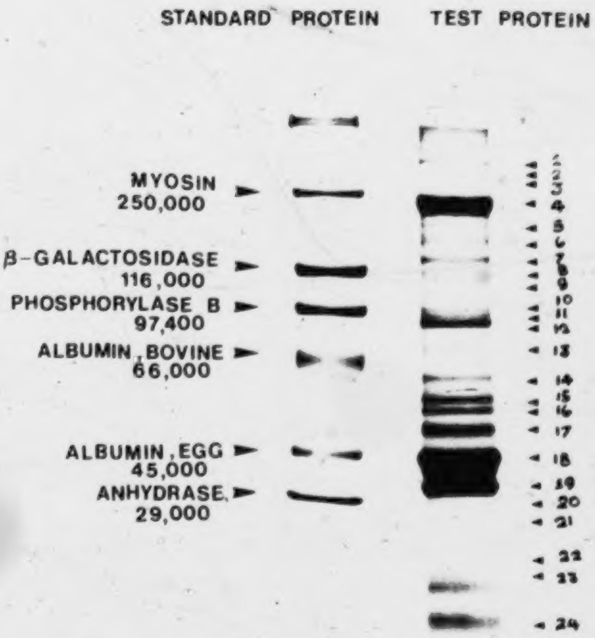

STANDARD PROTEIN
TEST PROTEIN
MYOSIN
250,000
β-GALACTOSIDASE
116,000
PHOSPHORYLASE B
97,400
ALBUMIN, BOVINE
66,000
ALBUMIN, EGG
45,000
ANHYDRASE
29,000
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

Figure 4.1 Electrophoretograms of the alkali-extracted protein from poultry deboning residues and standard protein (see Table 4.3 for identification of the numbered bands.

occurrence of proteins not only from meat muscles but also from the skin and bone marrow. They also suggested that the possibility of enzymic protein breakdown should be taken into consideration since the deboning process causes destruction of deboned material, which results in the liberation of many lysozomal enzymes. Also present was the protein subunit of molecular weight 17 X 10^3 daltons which may be attributed to the higher contents of the haem pigments originating from the bone marrow. The majority of the salt soluble proteins recovered in the bands were of molecular weight 47-38 X 10^3 and 21-17 X 10^3 daltons, from which they suggested the presence of the following proteins: actin, tropomyosin and the light chains of myosin and troponin and the degraded products of the heavy chains of myosin.

Since collagen, determined as hydroxyproline, was also present in the fresh protein isolate, it should be expected as one of the bands in the SDS-PAGE electrophoretogram. According to Weber and Osborn (1979), the allowable experimental error in the determination of molecular weight may be of the order of ±10%. Hence, it is possible that the subunit of molecular weight 93,800-110,000 daltons may represent an α chain of collagen (Figure 4.1). Nagai *et al.* (1964) estimated the molecular weight of the α-class of denatured collagen at 100,000, and that alkali-soluble collagen has been shown to comprise a high proportion of α-subunits (Kemp and Tristram, 1971). The extent at which collagen is present in soluble protein extracts is important by virtue of its possible effect on the nutritional and functional properties (Young and Lawrie, 1975a).

The low concentration of collagen in the alkaline protein extract from the poultry deboning residues (Table 4.1) may be attributed to the relatively low extraction temperature (22-24°C) used in the process. Solubilization of collagen takes place under mild hydrolysis (e.g. boiling in water, dilute acids, or alkalis) (Seifter and Gallop, 1966), wherein non-covalent bonds and some inter- and intramolecular bonds and a few main-chain peptide bonds are hydrolyzed (Hultin, 1985). This results in the conversion of the tri-stranded collagen structure to a more amorphous form, known as gelatin (Veis, 1965).

Aside from the poor nutritional quality characteristic of collagen, this fibrous protein seems to be less desirable for producing protein fibers. Huang and Rha (1974) stated that orientation of the protein chains cannot lead to crystallization unless the chains can fit into the lattice structure. The inherent bulkiness of proline and hydroxyproline can interfere in the process. Collagen is distinctive in containing about 12% proline and 9% hydroxyproline, an amino acid rarely found in other proteins (Lehninger, 1979).

4.1.6 Amino acid composition

The extent of intermolecular attractions that would occur during protein texturization is not only dependent on the size and shape of the protein molecules but also on the nature of the amino acids and their arrangement in the primary protein structure. It has been established that compositional factors are responsible for molecular associations involved in the cohesion phenomena of proteins (Wall and Huebner, 1981), as in the case of protein texturization. The presence of different amino acids in the deboning residue protein isolate (Table 4.4), would profoundly affect the nature of protein-protein interactions due to the formation of various chemical bonds or forces, as illustrated in Table 4.5.

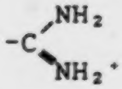
From the nutritional standpoint, the analysis had indicated the presence of the essential amino acids except tryptophan. The absence of tryptophan can be attributed to problems associated with quantitative determination of this amino acid residue in a protein, since acid hydrolysis usually results in the extensive destruction of this vital amino acid (Liu and Chang, 1971; Hugli and Moore, 1972). Spies and Chambers (1949) studied the factors affecting the stability of tryptophan in 5N NaOH and found that this amino acid is more labile when it is peptide-linked and that losses depend on the protein composition. In the present study, the proteins were subjected to alkali treatment using 5N NaOH (pH 10.5), which may have a damaging effect on

Table 4.4 Amino acid composition of the alkali-extracted, acid-precipitated protein from poultry deboning residues.

Amino Acid	nmoles/mg (± SD)[1]
Aspartic acid	507.66 ± 37.00
Threonine	433.23 ± 23.59
Serine	431.26 ± 40.26
Glutamic acid	1012.27 ± 57.93
Proline	436.51 ± 39.11
Glycine	664.68 ± 95.95
Alanine	676.09 ± 41.30
Cystine/2	67.63 ± 8.91
Valine	487.34 ± 33.02
Methionine	193.66 ± 21.09
Isoleucine	399.60 ± 29.34
Leucine	698.29 ± 35.69
Tyrosine	243.69 ± 18.03
Phenylalanine	328.43 ± 24.41
Hydroxyproline	53.87 ± 7.17
Lysine	575.00 ± 28.49
Histidine	204.64 ± 16.81
Arginine	408.93 ± 22.77

[1] Average values of three replicates.

Table 4.5 Structural forces in proteins[1].

Type	Interacting Groups	Example
Covalent Bond	C-C, C-N, C=O, C-H	Intra residue bonds
	C-N-C	Peptide
	S-S	Disulfide
Ionic Bond	$-NH_3^+$	α-Amino group
		Lysine
		Arginine
		Histidine
	NH+	Aspartic, glutamic
	-COO-	α-Carboxyl group
Hydrogen Bond	N-H...O=C	Amide-carbonyl group
	OH...	Serine, threonine, tyrosine
	NH..., NH_2...,	
	NH_3^+..., COO-	Polar side chains of residues
Van der Waals attractive force	Apolar groups	Apolar side chains
Electrostatic repulsive force	Polar groups of like sign	Polar side chains
Van der Waals repulsive forces	Steric hindrance between side chain groups	All groups

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[1] Taken in part from Jones (1964).

tryptophan.

Ozimek *et al.* (1985) compared the nutritional value of the mechanically separated poultry meat (PP) and alkali-extracted protein (EP) obtained from poultry deboning residues with casein, expressed as net protein utilization (NPU), protein efficiency ratio (PER) and true digestibility (TD). They found small but statistically significant differences between the NPU values of PP and EP, and both were significantly higher than casein. Significant differences were also observed in the PER between PP and the control casein, while TD of the extracted protein and casein was significantly different.

4.2 Effects of Freezing Mode and Temperature on Protein Texturization

4.2.1 Freezing Mode

As in other methods of protein texturization, fiber spinning and thermoplastic extrusion, freeze texturization is designed to fabricate a product which simulates some of the textural characteristics of meat. This refers specifically to the fibrous nature of the main meat protein components, myosin and actin, which are arranged in parallel direction as shown in Plate 4.1. The photomicrographs (Plate 4.1) show the longitudinal sections of a muscle; the muscle fibers (Plate 4.1a) and the myofibrils (Plate 4.1b), both in their natural parallel orientation.

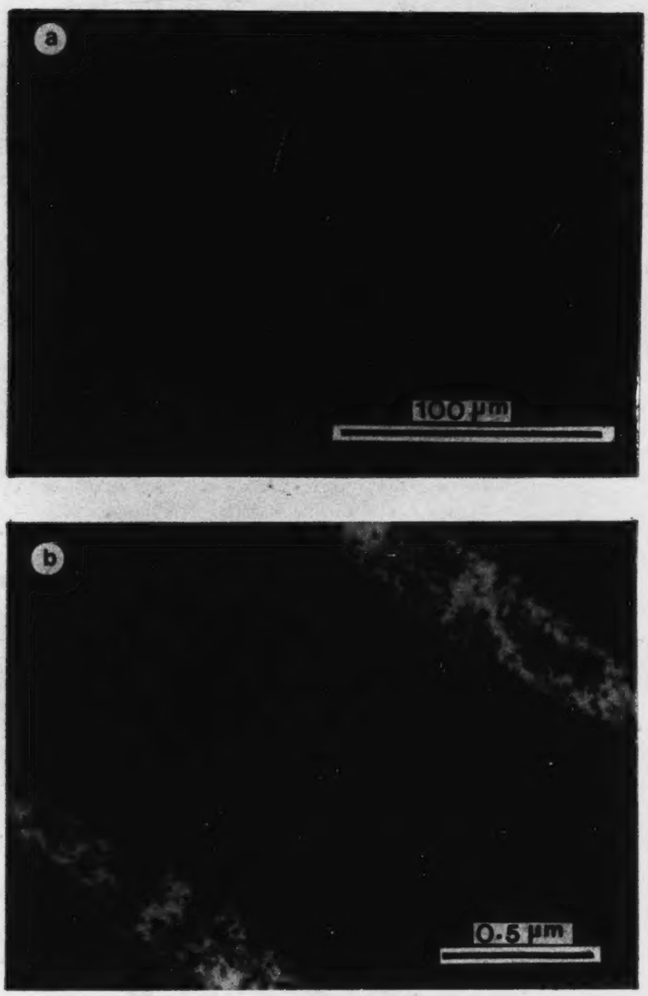
a
100 µm
b
0.5 µm

Plate 4.1 Electron micrographs showing longitudinal sections of a) muscle fibers (SEM) and b) myofibrils (TEM).

The texture of meat products is inherently dependent upon the fibrous nature of the meat (Kim and Lugay, 1978a). Similarly, the presence of a fibrous structure or parallel aligned protein mass is an important factor in simulating meat. The transformation of the original disorganized, paste-like protein slurry (Plate 4.2a) into a more organized structure (Plate 4.2b) could be attained by manipulating the direction by which heat is removed from the freezing system.

An investigation was conducted on the effect of the non-directional and unidirectional freezing modes on the texturization of protein. Results showed the presence of a "junction" inside the protein matrix, which was more distinct in the non-directionally freeze texturized protein slurry than in the unidirectional ones (Plate 4.3). The orientation of the protein mass as affected by the different freezing techniques can be depicted in the schematic diagram shown in Figure 4.2. In the non-directional mode, heat removal takes place in all directions and this was clearly demonstrated in the orientation of the protein sheets intersecting at the junction area. The ice crystals growing from the surface as "spears" (Lawrence *et al.*, 1985), exclude the proteinaceous slurry and solutes as the freezing process progresses. These solutes are concentrated between the ice crystals and in the direction of the freezing front, terminating at the geometrical center of the container. Hence, the junction can be assumed to contain a high concentration of protein and salts. A closer examination of

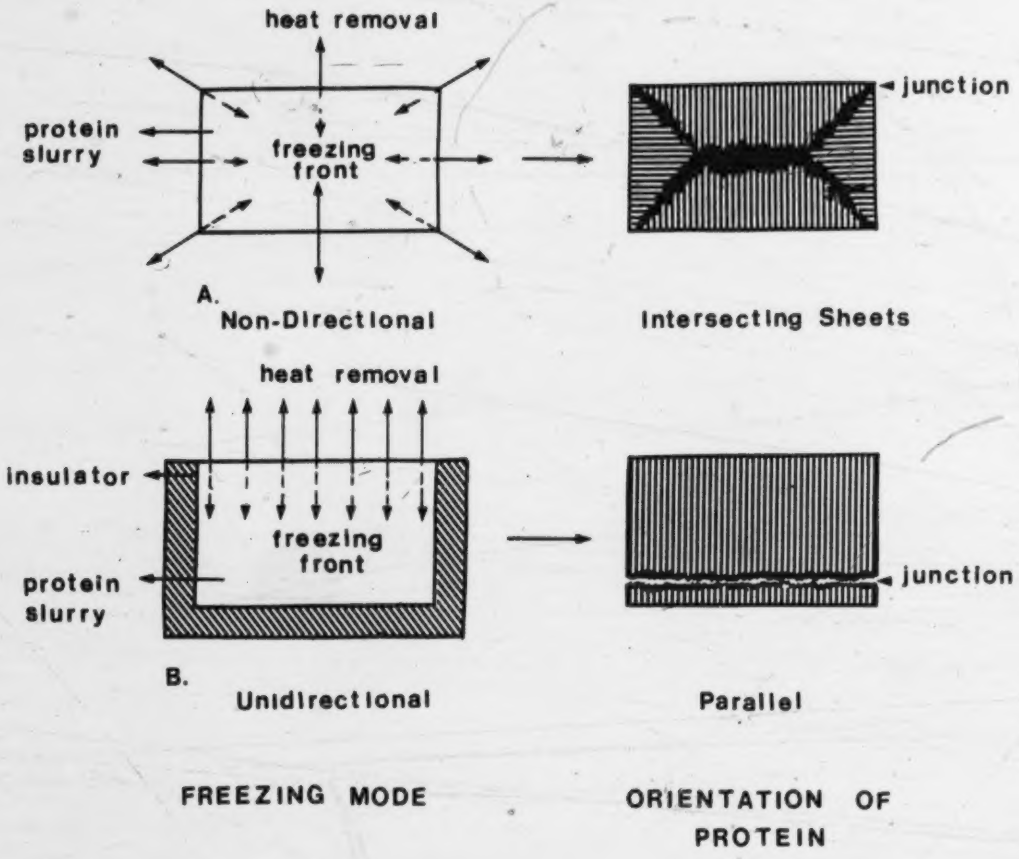
heat removal
protein slurry
freezing front
junction
A.
Non-Directional
Intersecting Sheets
heat removal
insulator
freezing front
protein slurry
junction
B.
Unidirectional
Parallel
FREEZING MODE
ORIENTATION OF PROTEIN

Figure 4.2 Schematic diagram of texture formation in a protein slurry following non-directional and unidirectional freezing modes.

a
10µm
b
10µm

Plate 4.2 Scanning electron micrograph of (a) the alkali-extracted, acid-precipitated protein slurry (pH 5) obtained from mechanically deboned poultry meat residues, and (b) protein slurry after unidirectional freeze texturization, showing fibrosity or texture formation.

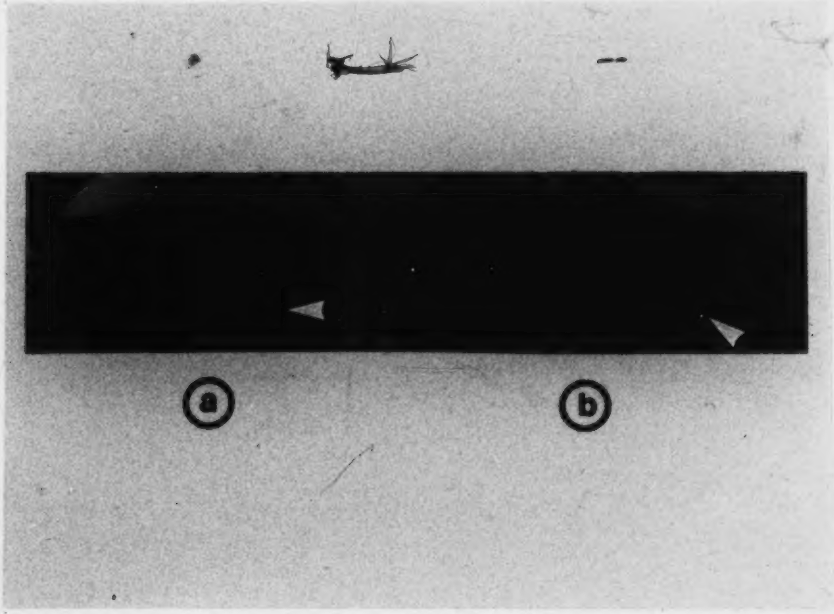

Plate 4.3 Unidirectional (a) and non-directional (b) freezing of protein slurry. Arrow shows the "junction" formed by the moving freezing front.

this area showed that the proteins are fused together (Plate 4.2), a possible indication of high salt concentration causing solubilization of the proteins. In contrast, unidirectional freezing produces parallel protein fibers all the way from the top to bottom (Plate 4.3, Figure 4.2), as a result of the heat removal from one direction. The not-so-distinct junction was present in the texturized protein but closer to the bottom, indicating the direction of the moving freezing front.

The force required to compress and extrude the texturized protein following the non-directional and unidirectional freezing techniques showed significant differences (Figure 4.3). The protein slurries texturized using the latter mode gave higher compressive force values than the former. This result suggests the positive role of the more parallel aligned protein fibers in providing greater strength to the texturized product. On the other hand, the presence of the junction, representing the dissolved, untexturized protein in the non-directionally frozen samples might have contributed to the weakening of the structure of the entire protein matrix. The results had indicated also that there was no significant difference in the compressive force values between the unidirectional and non-directional freeze texturized protein slurry at pH 5 and at -25°C.

The freezing technique seemed to have an additional effect on the texture of the protein under the alkaline pH

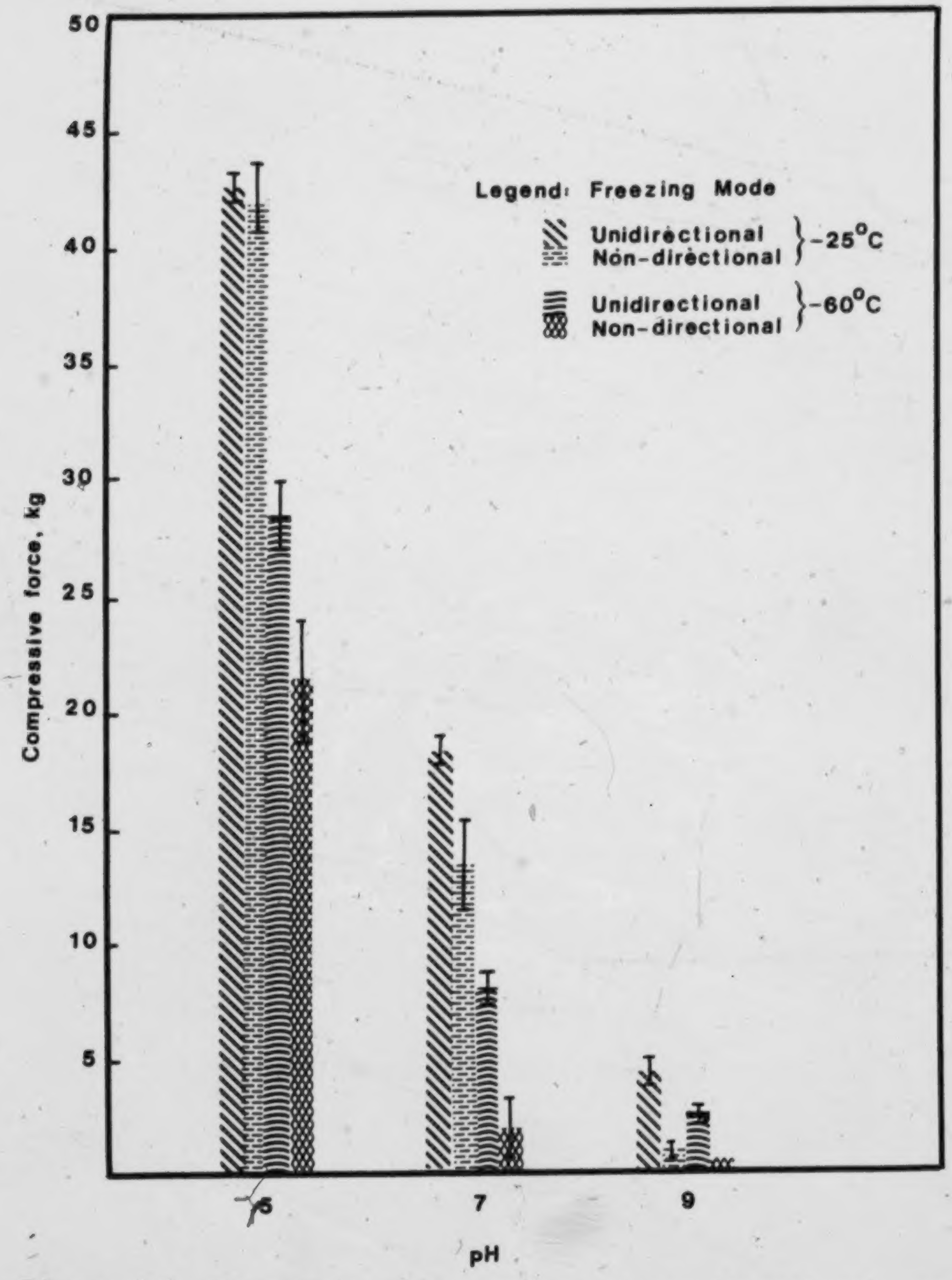

Figure 4.3 Compressive force values of non-directional and unidirectional freeze texturized protein slurry and different pH's and temperatures. (Horizontal bar = ± SD).

and lower freezing temperature. There was greater reduction in the compressive force values in the non-directionally freeze texturized protein compared to the unidirectional ones. Since texture is also governed by the structural arrangement of the protein matrix, a close analysis of the microstructures reveals presence of the dendritic or branched orientation of the protein sheets, which was predominant feature in the non-directionally frozen protein slurry (Plate 4.4a). Comparison of the non-directional and unidirectional technique showed relatively greater reduction in thickness of the sheet structures as the pH of the protein slurry was increased, following the former freezing technique (Plate 4.4).

Freezing the protein slurry by cooling according to a defined directional pattern results in well-defined, well-ordered fibrous or sheet structures produced by elongated ice crystals (Plates 4.5 and 4.6). Similar results were obtained by Kim and Lugay (1977) in freeze texturizing protein materials, such as soy milk, soy isolates, meat slurries, fish slurries and other heat coagulable proteins. These authors also noted that the protein sheets are not completely independent of each other but are joined at sufficient locations to bind the individual sheets into a branched or cross-linked structure, resulting into a cohesive mass. These interconnections of the parallel protein sheets are also evident in Plate 4.6a. The degree of binding achieved is just sufficient to provide cohesiveness

Plate 4.4 Scanning electron micrographs of non-directionally freeze texturized protein slurry: a) pH 5, -25°C; b) pH 5, -60°C; c) pH 7, -25°C; d) pH 7, -60°C; e) pH 9, -25°C; f) pH 9, -60°C.

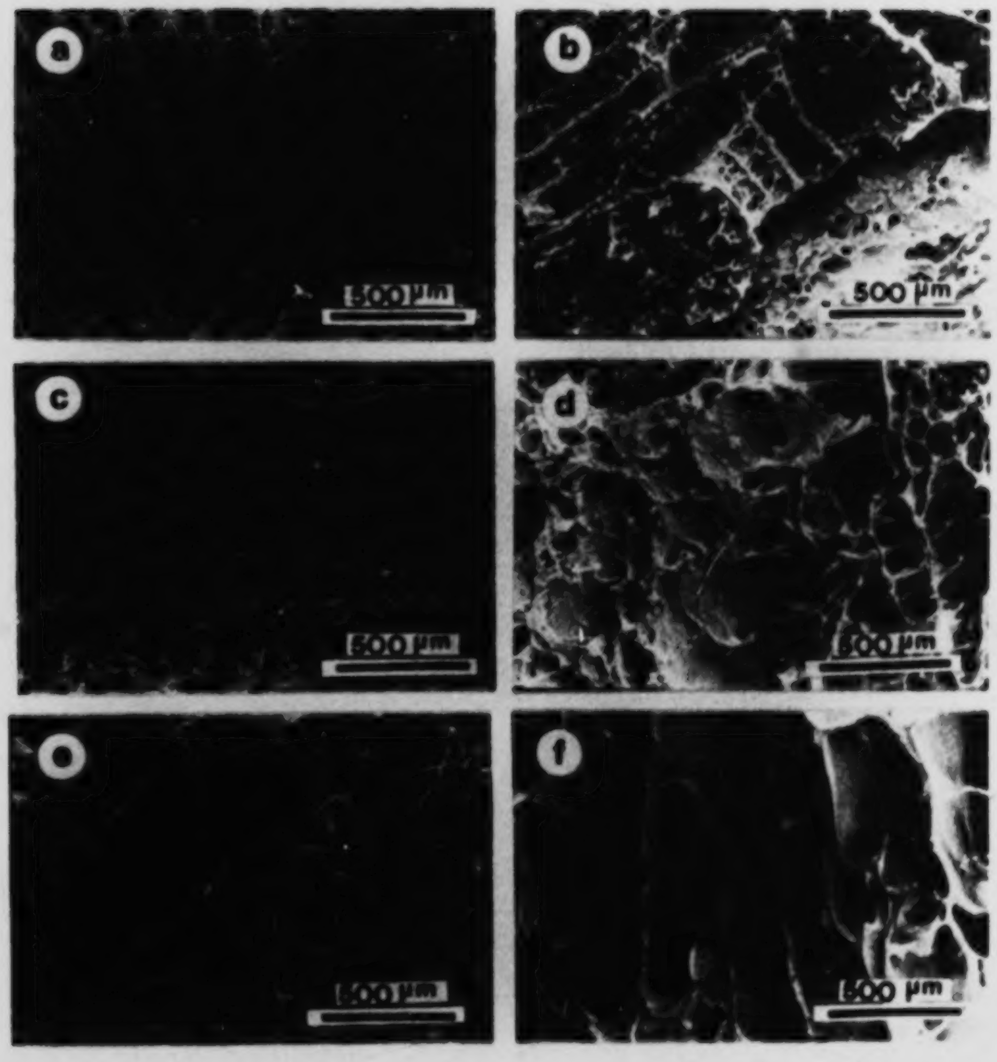
a
500 µm
b
500 µm
c
500 µm
d
500 µm
e
500 µm
f
500 µm

Plate 4.5 Unidirectionally freeze texturized protein slurry (pH 5, -25°C) showing the protein sheet structures.

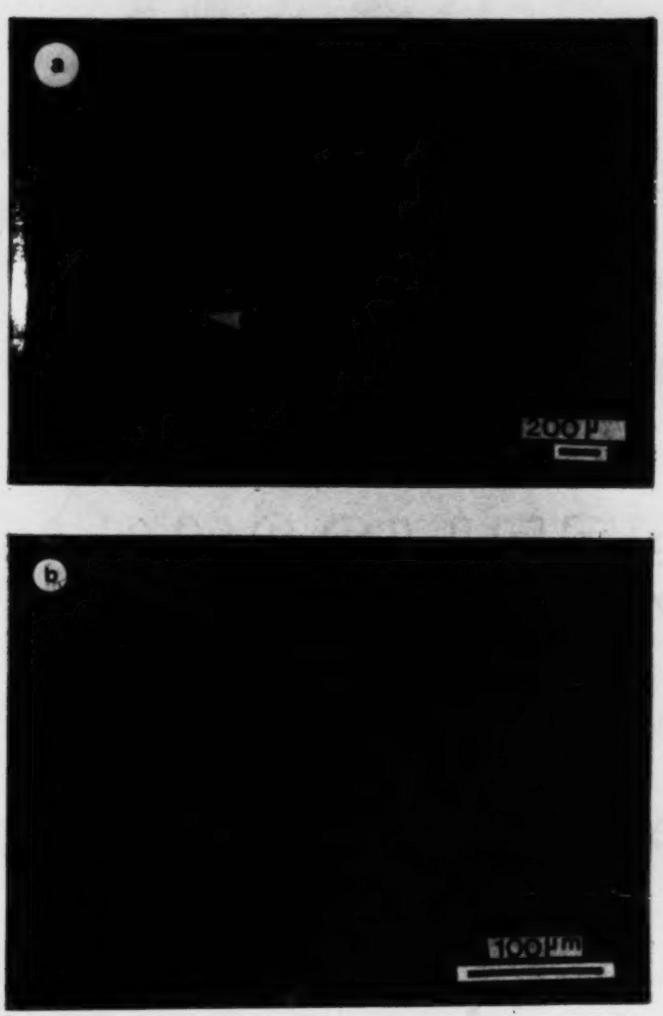

Plate 4.6 Unidirectionally freeze texturized and heat-set protein slurry (pH 5, -25°C): (a) showing the main and interconnecting protein sheet structures (arrow), and (b) a single sheet showing compactness and homogeneity.

to the final product similar to cooked meat, and does not destroy the substantially independent sheets (Kim and Lugay, 1977). This binding, achieved during the formation of the sheet structures, eliminates the need for added binder materials.

A closer analysis of the cross-section of the protein sheet structure (Plate 4.6b) reveals the compactness and homogeneity of the protein mass. This provides evidence of the compression that had taken place during the freeze texturization process. There is a distinct contrast in appearance between the freeze texturized protein and the original protein paste (Plate 4.2a), demonstrating the transformation of the level of organization of the protein as a result of freezing. At this point, it appears that the mechanical effect (compression) provided by the growing ice crystals gave a considerable amount of cohesive forces that enabled the texturized protein to retain its structural integrity during thawing at room temperature and heat setting.

The results obtained in this part of the study agree with the suggestions of numerous workers that the desired parallel orientation of the texturized protein mass, which simulates striated muscle, can be obtained following the unidirectional freezing technique (Middendorf *et al*., 1975; Kim and Lugay, 1978a,b; Lawrence, 1981).

4.2.2 Freezing temperature

The mechanism of fiber formation induced by freezing is due to the physical casting of proteinaceous fibers caused by the ice crystal growth (Lawrence *et al.*, 1985). The size, number, alignment and, to some extent, the characteristics of the fibers are directly dependent on the size, number, nature and alignment of the ice crystals. The first three characteristics of the ice crystals are governed by the rate of freezing or freezing temperature.

After non-directional freezing at -25°C (medium-fast freezing), the protein sheet structures appeared relatively thicker and firmer compared to those protein slurries frozen at -60°C (fast freezing). Also, the protein mass exhibited puffiness under the lower freezing temperature (Plates 4.4b,d,f). These differences in surface topography and structural orientation were reflected in the significant differences in the compressive force values exerted by the texturized protein (Figure 4.3). On the other hand, a very evident effect of freezing temperature was demonstrated when the protein slurry was frozen at -196°C using liquid nitrogen. The rapid freezing resulted in a highly porous or spongy and randomly oriented protein matrix (Plate 4.7). The small voids represent the areas formerly occupied by the numerous ice nuclei formed by the rapid freezing (Fennema *et al.*, 1973).

Hashizume (1978) determined the effects of freezing temperatures on the texture of kori-tofu. He found that at

Plate 4.7 Non-directionally freeze texturized protein slurry at pH 5, -196°C (using liquid nitrogen).

-5°C large ice crystals were produced, resulting in very coarse textures. Rapid freezing at -70°C produced very minute ice crystals, leaving very small pores after thawing; at this state the product was considered untexturized. At -25°C, relatively small ice crystals were produced, giving a completely spongy texture. Freeze texturing at -5°C yielded a hard kori-tofu while that obtained at -70°C was soft and fragile. Hashizume concluded that a texturizing temperature of -20°C is appropriate for obtaining the spongy protein intended as a substitute for animal meat.

4.3 Effect of Alkali and Acid Treatments on Fiber Formation

Greater understanding of the texture formation in the amorphous protein isolates during texturization process should be gained by taking into account the pretreatments given to the intact proteins, since many conformational changes in the protein structure take place at this stage. As mentioned earlier, the alkali treatment of the mechanically deboned poultry meat residues solubilizes both the myofibrillar and sarcoplasmic components of the muscle proteins. Upon acidification of the alkali extract from pH 10.5 to 5.0, the myofibrillar proteins are mainly precipitated while the sarcoplasmic proteins remain in the supernatant solution (Lawrence, 1981). However, the latter proteins may remain with the myofibrillar coagulation in the protein isolate after centrifugation at low speed.

The occurrence of quite large molecular weight components in the protein isolate is a clear indication that it satisfies the raw material requirement for texturization (Hartman, 1978; Shen and Morr, 1979; Huang and Rha, 1974). However, it is not yet fully established if high molecular weight polypeptides would affect the texturization in the case of the freeze-alignment process.

The presence of protein interactions leading to fiber formation and extended cross-linking in the acid-precipitated, unfrozen protein isolate can be clearly demonstrated by scanning electron microscopy (Plate 4.8a). This result strongly depicts the occurrence of protein interactions during the acidification of the alkali extract from pH 10.5 to 5.0; needed for the isoelectric precipitation. The presence of sulfhydryl groups in the protein mixture (Table 4.1) would suggest possible protein polymerization via the disulfide bond formation, to some extent, occurring especially under the alkaline region during pH adjustment. This was indicated when the unfrozen protein samples for electrophoresis were dissolved in the sample buffer containing 2-mercaptoethanol, which showed relatively higher solubility of the protein than in the absence of this reducing agent. Similar interpretation by Kelley and Pressey (1966) postulated that alkaline conditions favor disulfide bond formation, while acidification brings many polypeptide chains together, favoring hydrogen and ionic bonding.

Plate 4.8 Scanning electron micrographs showing fiber formation and extended cross-linking in a) fresh alkali-extracted, acid-precipitated protein isolate and b) freeze texturized, freeze dried protein.

The protein network similar to that in the unfrozen protein (Plate 4.8a) was also observed in the freeze texturized, freeze dried protein as shown in Plate 4.8b. It appears that fiber formation and extended cross-linking following the alkali and acid treatments could be preserved and probably promoted during freeze texturization. Hashizume *et al.* (1971) postulated two events that could take place during this process. First, ice crystal formation during freezing brought the protein close together, thereby concentrating the fibers between the parallel oriented ice crystals. Second, further protein-protein interactions may have occurred at the early stages of the freezing process in the unfrozen concentrated solution.

Connell (1968) stated that interfilament reactions profoundly affect texture, and it therefore seems likely that similar reactions are a main contributor to the toughening occurring during frozen storage of fish, which is attributed to myofibrillar changes. The gradual formation of permanent interfilament cross-links via the cross-bridges of the thick filaments could explain the progressive reduction in both interfilament distances. Cross-links of this type would be mostly between either native or denatured myosin molecules and native actin molecules (Connell, 1968). Although the foregoing events may take place in the intact muscle tissue, similar reactions could be assumed in the case of freezing of protein isolates. In both systems, freezing causes concentration of the proteins and solutes, providing conditions for myofibrillar changes.

Based on the electron microscopic analyses done in this part of the investigation and considering the other works on the behaviour of myofibrillar proteins at sub-zero temperatures (Oguni *et al.*, 1975; Ohnishi *et al.*, 1978; Matsumoto, 1979; Jarenback and Liljermark, 1975a,b), it could be concluded that fiberization and cross-linking of myofibrillar filaments are initiated during the pretreatment steps of the protein texturization process.

4.3.1 Cross-linking mechanisms in freeze-texturized protein

In the present study, the nature of crosslinking in the protein isolate subjected to various stages of freeze texturization was determined by measuring total protein solubility in different solvents. This approach was adopted from Matsumoto (1980), who determined the nature of cross-linking of isolated carp actomyosin or myosin in 0.6M KCl or 0.05M KCl and stored at -25°C. The following solutions were used in the present study: water (to test for nonspecific association forces), 0.6M KCl (to test for ionic bonds), 1.5M urea (to test for hydrogen bonds), 8M urea (to test for hydrogen and hydrophobic bonds), 1M KOH (to test for ionic bonds and others), and 1% SDS (to test for all possible forces except disulfide bonds).

The solubilities of the protein at the different stages of the texturization process, in various test solutions are shown in Table 4.6. The differences in solubilities of the

Table 4.6 Solubility[1] of alkali-extracted, acid-precipitated proteins obtained from poultry deboning residues following texturization treatments.

	Nature of Protein Isolate[2]		
Solvent	Fresh	Thawed, freeze texturized	Heat-set, freeze texturized
Distilled water	1.89d	2.87f	0.55c
0.6M KCl	8.45c	14.80d	1.74c
1.5 M Urea	2.16d	5.44e	1.20c
8M Urea	12.67b	20.59c	5.98c
1M KOH	68.17a	96.24a	93.62a
1% SDS	65.47a	83.39b	15.72b

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[1] Expressed as mg/100 ml thawed liquid.
[2] Figures in the columns followed by the same letter are significantly different at $P \leq 0.01$.

proteins in each test solution are highly significant ($P \leq 0.01$). High solubilities were exhibited in 1M KOH and 1% SDS, with 8M urea and 0.6M KCl indicating lower solubilities, while the proteins were least soluble in distilled water and 1.5M urea.

Following the interpretation advanced by Matsumoto (1980) regarding the solubility of carp actomyosin in the same test solvents, similar statements can be drawn from the results obtained in the present experiment. Comparing protein solubilities in distilled water, the presence of KCl, KOH or SDS, which had indicated increased solubility, showed that aggregation of the protein molecules in the fresh isolate was predominantly due to ionic bonding. The differences in solubility between 1.5M urea (test for hydrogen bond) and 8M urea (test for hydrogen and hydrophobic bonds) resulted from the disruption of the hydrophobic bonds at the higher urea concentration. After freezing and thawing, there was a general increase in the protein solubility in all of the test solvents. This result suggested possible alterations in the protein molecules, leading to additional protein interactions through hydrogen, hydrophobic and ionic bonding, as a result of freeze texturing the protein. The increased chemical bonding seemed to have provided the increased cohesiveness in the texturized protein. However, heat-setting the freeze texturized protein gave highly significant ($P \leq 0.01$) decreases in the protein solubility, indicating stabilization of the various chemical forces brought about by this thermal treatment. Moreso, it was necessary to stabilize or fix (by heat setting) the parallel oriented protein sheets after thawing at room temperature, since these structures could easily disintegrate during handling. This is another indication of the presence of weak bonds in the freeze texturized protein.

Since the protein isolate or slurry was prepared by isoelectric precipitation, there is no doubt that the protein molecules are held together mainly by ionic bonds. Other forces, such as hydrogen or hydrophobic bonds, also contribute to some extent. Based on this solubility test and electron microscopic observations, it could be concluded that the freezing process has a two-fold effect. One, the growing ice crystals concentrate the protein and other solutes in the unfrozen liquid portion, allowing possible alterations in the protein conformation, leading to additional intra- and intermolecular reactions. Second, these protein interactions, coupled with the compressive force provided by the unidirectionally growing ice crystals, yielded a strongly cohesive and parallel oriented, texturized protein mass held together by ionic, hydrogen and hydrophobic bonds.

4.3.2 Texturization of mechanically deboned meat paste

The meat paste used in this study was obtained by a mechanical deboning process, involving crushing or pregrinding chicken necks and backs or frames, and expressing them through a sieve. Meat (or meat paste) passes through the sieve and is thus separated from the bone residue (Dawson and Gartner, 1983).

An attempt was made to texturize mechanically deboned poultry meat paste to determine if it would exhibit behaviour similar to the protein isolate. The protein isolate used in this part of the study was obtained from the same meat paste, also by aqueous alkaline solubilization, acid precipitation method. The proximate composition of the meat paste and the protein extract is presented in Table 4.7.

Results of freeze texturization (unidirectional) showed that the meat paste did not texturize as the alkali, acid-treated protein. The meat paste remained in its original mashy, untextured state after the freeze-thaw process. In contrast, the texturized protein isolate showed the parallel protein sheet structures, the texture formation characteristic of the unidirectional freeze-alignment technique (see Plate 4.3).

The failure of the meat paste to texturize seemed to indicate the role of the alkali treatment and possibly also of the interferring substances in the freezing system. Table 4.7 shows the chemical composition of the meat paste and the protein slurry. The high fat content (22.56%) in meat paste might be one of the contributing factors relating to this freezing response. Since texturization requires protein-

Table 4.7 Chemical composition and pH of the fresh, mechanically separated meat paste and alkali-extracted protein[1].

Product	Water (%)	Protein (%)	Fat (%)	Ash (%)	pH
Meat Paste	66.84	9.94	22.56	0.63	6.62
Alkali Extracted Protein	88.46	6.08	5.05	0.44	5.40

[1] From Ozimek *et al*. (1985). The samples used in this study were taken from the same batch.

protein interactions (Boyer, 1954; Kelley and Pressey, 1966; Hashizume *et al.*, 1971) to promote or generate an organized structured protein matrix, this process necessitates that the protein be drawn closer together. Such myofibrillar protein interactions are needed to provide good coherence in the protein matrices to maintain the structural integrity of the texturized product. However, this could have been prevented by the presence of the large amount of fat among the protein molecules, which were concentrated in the unfrozen liquid portion of the freezing system. Another important factor that could have affected the proteins in the original meat paste was the absence of alkali and acid treatments. The effects of alkali in proteins have been well discussed with respect to texturization in the other sections of this work. The results obtained in this experiment emphasize the important roles of the solubilization and denaturation through alkali treatment, and aggregation of the proteins at isoelectric precipitation, in protein texturization. Such treatments provided suitable conditions for possible interactions between extended polypeptide chains in order to promote fiberization and thus achieve protein texturization.

The results obtained in this part of the study were not consistent with the patent claims made by Middendorf *et al.* (1975), who were able to freeze texturize animal protein materials with fat content up to 10%, but without the alkali treatment. These authors stressed, though, that the slurry should be uniform and the protein reduced to a small enough particle size to resemble a uniform emulsion, for example, if a high percentage of water insoluble material such as fats and oils is employed. On the other hand, Kim and Lugay (1978a) indicated that addition of excessive amounts of fat to the protein system would be undesirable due to reduction of the tensile properties of the protein fibers. In other cases, a reduced tensile strength would be desirable if it would impart a more tender texture to the product (Kim and Lugay, 1978a).

Further investigations are obviously needed in order to ascertain the effects of other substances (e.g. fats, carbohydrates) on the freeze texturization behaviour of myofibrillar proteins. In particular, the protein-lipid interactions would be important to investigate in view of the flavour and texture imparted by fat, if the overall textural and sensory characteristics of natural meat are to be considered in the freeze texturized protein product.

4.4 Chemical Modification of the Protein Slurry before Freezing and its Effects on Freeze Texture Formation

The protein isolate used in this study was obtained by isoelectric precipitation of the proteins from the aqueous alkaline extract. As mentioned in other parts of this study, maximum ionic bonding and, to some extent, hydrogen and hydrophobic bonding may be responsible for maintaining the structural integrity of the textured protein mass. In this segment of the investigation, pH adjustment and addition of calcium or urea into the protein slurry raw material were made in an attempt to disrupt the forces holding the protein molecules together and to investigate the texture forming mechanism. The behaviour of the poultry meat proteins under these conditions was studied in terms of solubility, macro- and microstructure and texture strength of the freeze texturized product. The concept forwarded by DeMan (1976), who stated that texture could be viewed as a direct consequence of microstructure which in turn originates from chemical composition and physical forces, was pursued in this study.

4.4.1 Amount of soluble protein after freezing

4.4.1.1 Effect of pH

The liquid that separated during thawing at room temperature of the freeze texturized protein was analyzed for protein content. The amount of protein dissolved in the thawed liquid was used to assess the extent of protein-protein interactions following the different pH and calcium or urea treatments of the protein slurry at -25 and -60°C freeze texturization temperatures. Minimum amounts of soluble proteins were observed in products texturized at pH 5 both at -25 and -60°C (Figures 4.4 and 4.5). This was expected since protein molecules at their isoelectric point experience maximum attraction. Hydrogen or electrostatic bonds should be maximal around the isoelectric point of the

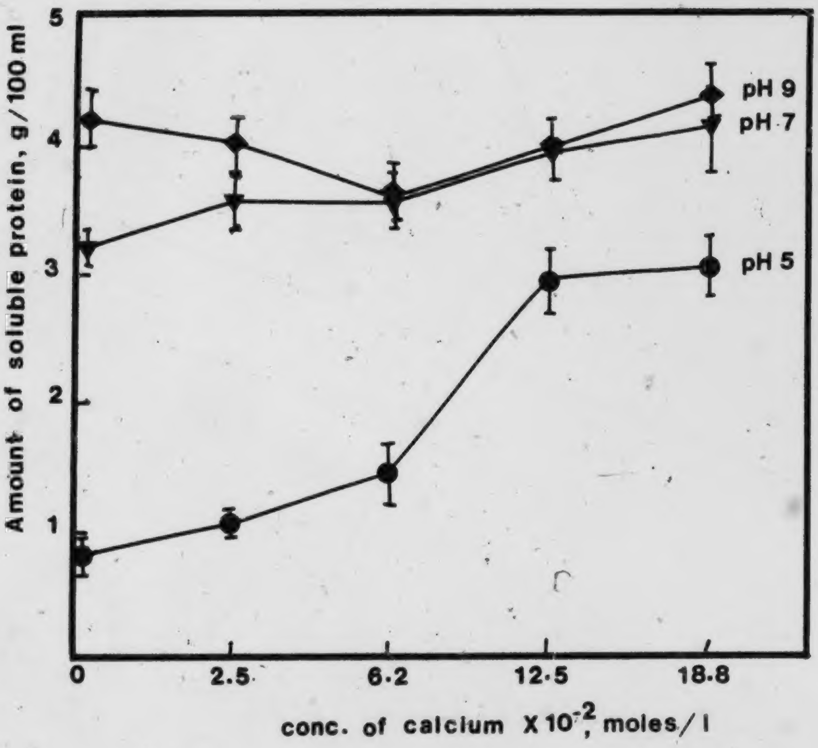

Figure 4.4 Amount of soluble poultry deboning residue protein in the thawed liquid after freeze texturization at different pH's and concentrations of calcium at -25°C.

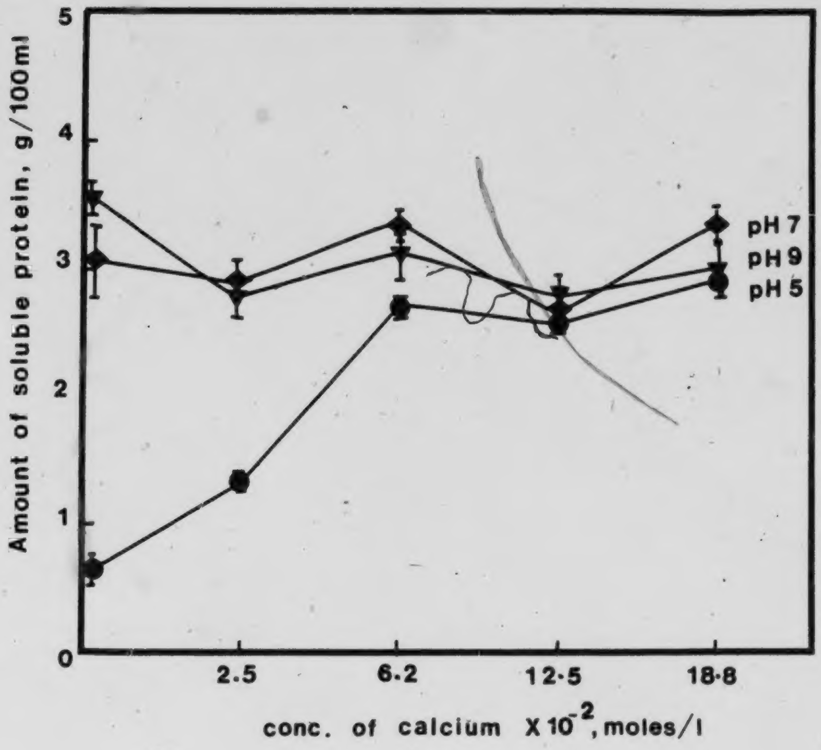

Figure 4.5 Amount of soluble poultry deboning residue protein in the thawed liquid after freeze texturization at different pH's and concentrations of calcium at -60°C.

protein (Hamm, 1960). Hence, the protein molecules have the least tendency to migrate to the solution (thawed liquid), as indicated by the lowered amount of soluble protein. Increasing the alkalinity of the freezing system (protein slurry) resulted in a highly significant ($P<0.01$) increase in the amount of protein that solubilized in the thawed liquid. Statistical analysis showed that amount of soluble protein at pH 7 or 9 was significantly higher ($P<0.01$) than at pH 5. However, there were no significant differences in the soluble protein values arising from pH 7 and 9 treatments. This result suggested that, even though there was concentration of the proteins in the unfrozen liquid portion between the parallel ice crystals formed during freezing, the presence of repulsive forces created by the excess negatively charged hydroxyl ions (OH^-) surrounding the protein molecules prevented their aggregation.

There was a significant reduction ($P<0.05$) in the amount of soluble protein in the thawed liquid at lower temperature and under the alkaline conditions (Figure 4.4 vs 4.5). The additional aggregation of the protein, thereby reducing the amount that dissolved in the thawed liquid, might be attributed to the effect of lower freezing temperature. It is possible that the cross-linking of the proteins leading to denaturation and aggregation is of a secondary type formed between the apolar regions of adjacent molecules (Connell, 1968). Further, Connell stated that these regions are normally buried in the interior of the molecule but are brought to the surface as a result of denaturation, due to decreased temperature.

4.4.1.2 Effect of added calcium

As mentioned earlier there was an increase in protein solubility toward alkalinity. A similar observation was also noted in the protein slurry at the isoelectric point (pH 5) upon addition of increasing amounts of calcium (as $CaCl_2$) both at -25 and -60°C (Figures 4.4 and 4.5). The maximum attraction experienced by the protein molecules at pH 5 appeared to be disrupted in the presence of this neutral salt, as reflected in the increased amount of solubilized protein. This weakening of the forces holding the protein molecules together at pH 5 may have been due to the screening effect of the chloride ions on the positively charged groups on the protein molecules (Hamm, 1960). Calcium binding may also be possible at pH 5 (4.0 μmoles Ca per g structural proteins of beef muscle; Hamm, 1959). However, there could still be repulsive forces in the protein slurry, enough to cause some protein fragments to go into the thawed liquid.

Statistically, the amount of protein dissolved in the thawed liquid differed significantly ($P<0.01$) due to the effects of pH, temperature and concentration of added calcium and the interactions of these factors. Under higher pH conditions (pH 7 and 9; Figures 4.4 and 4.5), small increments in the amount of solubilized proteins were noted at different concentrations of calcium. This may be due to the limited calcium binding of the proteins, hence indicating the limited binding sites on the protein molecules. Added calcium ions attach to the protein by electrostatic bonds and are more loosely bound than the calcium of the complex Ca-protein compound but more tightly than alkali metal ions (Hamm, 1960). The content of calcium in the structural proteins of beef muscle, post-mortem storage for 5 days at pH 7, was found to be 6.5 μmoles Ca/g protein (Hamm, 1959).

4.4.1.3 Effect of added urea

In the preliminary studies, concentrations of urea greater than 1 mole/l and under alkaline pH's, yielded a highly viscous protein slurry which remained in that state after the freeze thaw process. This showed that totally solubilized protein could not be texturized. Thus, concentrations up to 0.7 moles/l urea were employed, which gave partially solubilized protein slurry. This was consistent with the findings of Lillford (1985). Also, in the manufacture of "yuba" film, a soybean food product in Japan, it was found that the soy protein film did not form beyond pH 10.5 because of the intermolecular repulsion of the negative charges of protein. Moreso, addition of urea beyond 1M of N-ethylmaleimide (NEMI) to the protein solution markedly disturbed the film formation, suggesting that intermolecular hydrogen bonds, hydrophobic linkages or disulfide bonds contribute to the insolubility of the film (Okamoto, 1978).

Minimum solubilities were observed in proteins solubilized at pH 5, both at -25 and -60°C, in the presence of urea (Figures 4.6 and 4.7). However, there were no significant differences in this parameter with increasing amount of urea in the protein slurry.

Increasing the pH of the protein slurry from 5 to 9 yielded highly significant differences ($P<0.01$) in solubility, both at -25 and -60°C (Figures 4.6 and 4.7). The solubilizing activity of urea is considered to be well-favored under alkaline conditions (Lapanje, 1979). Again, statistical analysis showed no significant differences in the protein solubility values due to the concentration of urea.

The denaturing and solubilizing effects of urea, specifically by disrupting the hydrogen and hydrophobic bonds (Lundgren, 1949), differ according to its concentration. A strong concentration of urea (8M) is needed to disrupt effectively the hydrophobic and hydrogen bonds, while at low concentration (1.5M) only the hydrogen bonds are affected. Based on the low concentrations of urea used in the present study, it could be assumed that only the hydrogen bonds were broken. The increased alkalinity in the freezing system dissolves the protein by disrupting both the ionic and hydrogen bonds. As mentioned earlier, hydrogen bonding is maximal at the isoelectric point (pH 5), while such bonds can be cleaved at low and high pH. In the presence of a low amount of urea, the remaining hydrogen

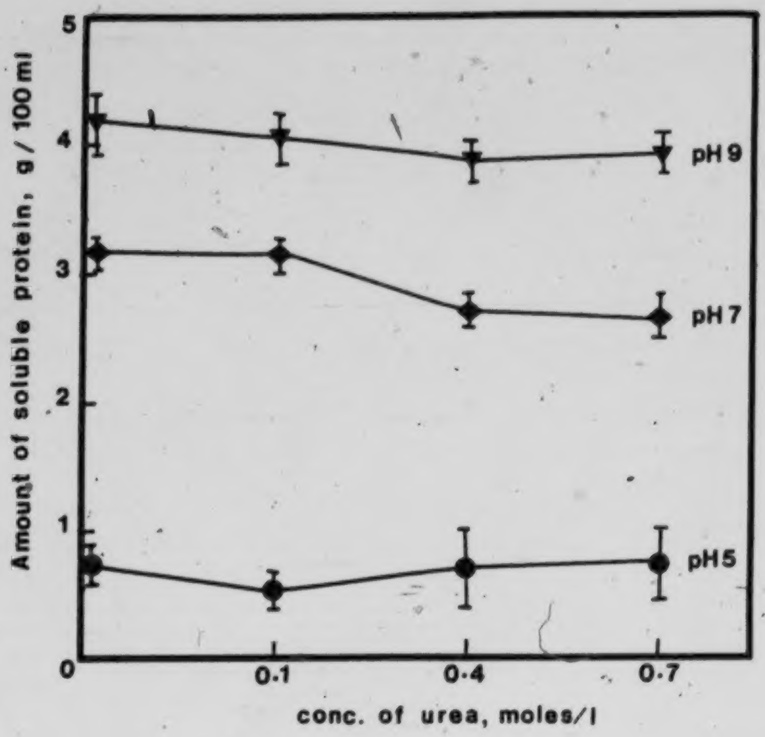

Figure 4.6 Amount of soluble poultry deboning residue protein in the thawed liquid after freeze texturization at different pH's and concentrations of urea at -25°C.

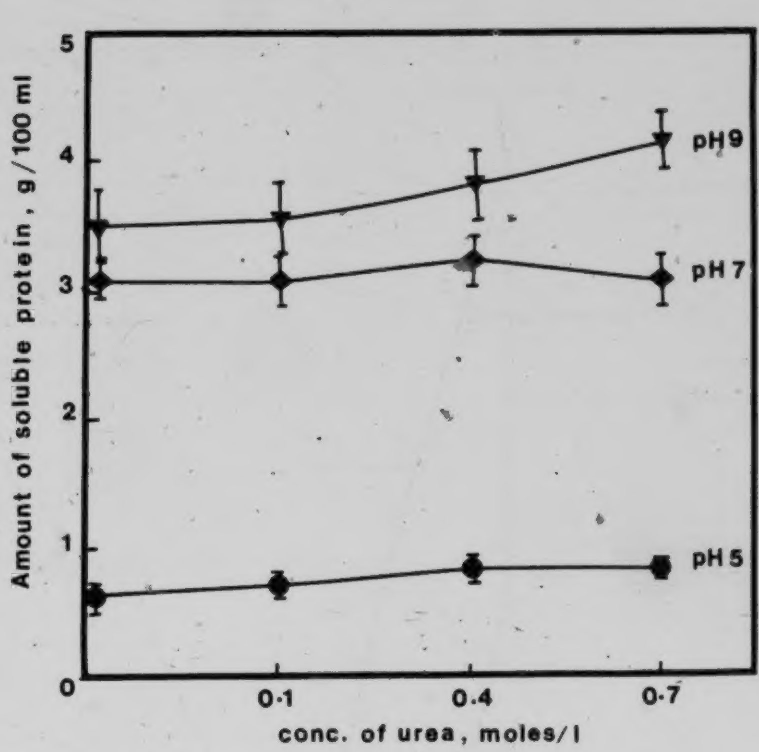

Figure 4.7 Amount of soluble poultry deboning residue protein in the thawed liquid after freeze texturization at different pH's and concentrations of urea at -60°C.

bonds (possibly a small number) could be disrupted. This could explain the insignificant differences in the protein solubility even as the amount of urea increased in the protein system.

4.4.2 Macro- and microstructural analyses

4.4.2.1 Effect of pH

A greater understanding of the mechanism of texture formation has been made possible through studies of microstructure formation (DeMan, 1983; Kazemzadeh *et al.*, 1982; Aguilera *et al.*, 1976; Maurice *et al.*, 1976). Using micrographs, texture formation within a product can be evaluated at various steps of texturization. Further, micrographs have been useful in assisting researchers to develop theoretical models for the behaviour of macro- and microstructures within a product, for example, during extrusion and fiber spinning (Kazemzadeh *et al.*, 1982). Published microstructures of freeze texturized protein products are almost nil.

Differences in the macro- and microstructures of the freeze texturized protein slurries were mainly due to the pH treatments (i.e., pH 5, 7 and 9). The more prominent parallel oriented protein sheets characteristic of the protein slurry freeze texturized at -25°C was observed in product at pH 5 (Plates 4.9a and 4.10a). The overall structure appeared firm and almost devoid of microcavities in the main protein sheet and interconnecting structures as

Plate 4.9 Photomicrographs of freeze texturized protein slurry at -25°C: a) pH 5, 0 ppm Ca; b) pH 7, 0 ppm Ca; c) pH 9, 0 ppm Ca; d) pH 5, 1.88 x 10^{-1} moles/l Ca; e) pH 7, 1.88 x 10^{-1} moles/l Ca; f) pH 9, 1.88 x 10^{-1} moles/l Ca.

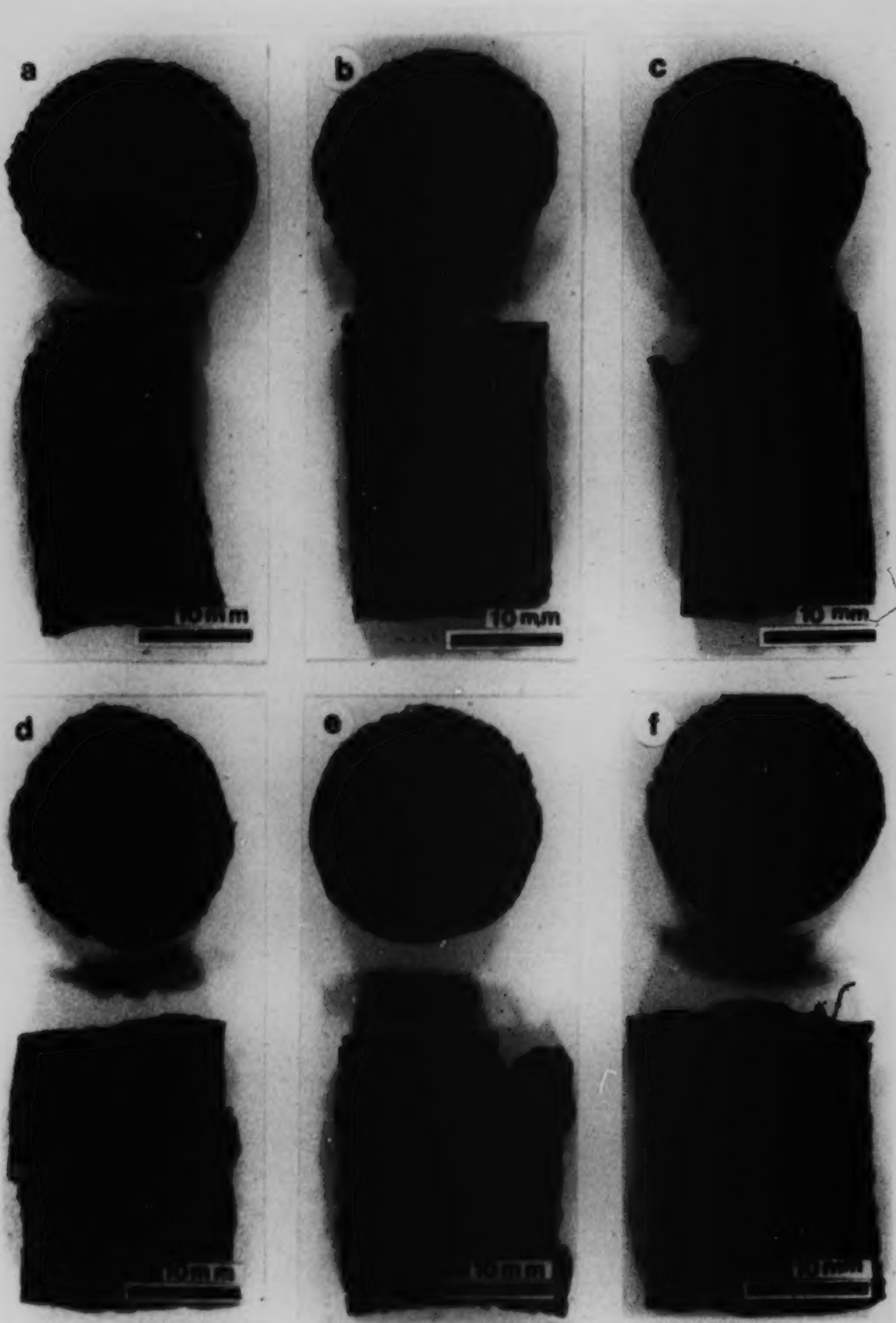
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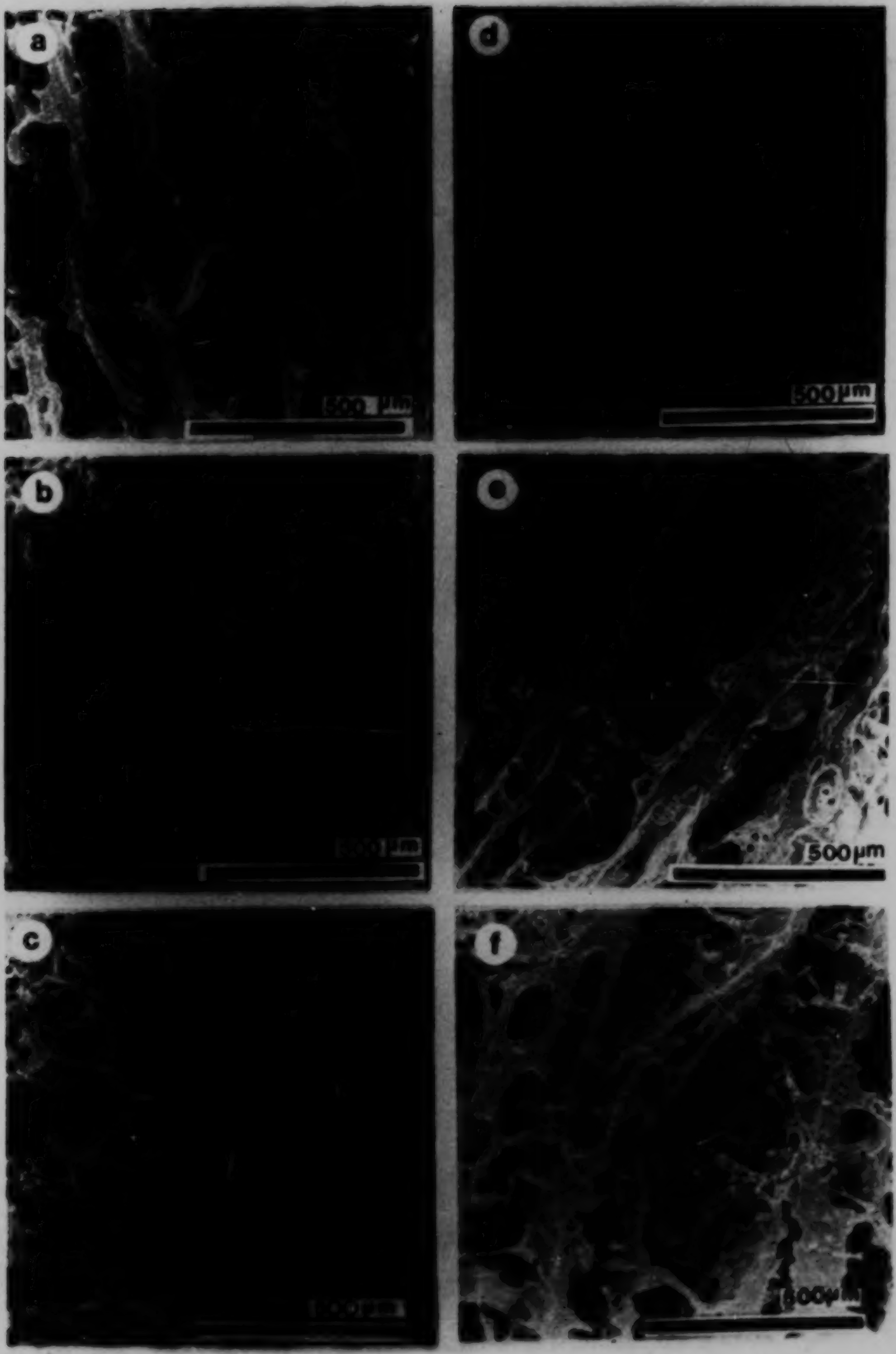
Plate 4.10 Scanning electron micrographs of freeze texturized protein slurry at -25°C: a) pH 5, 0 ppm Ca; b) pH 7, 0 ppm Ca; c) pH 9, 0 ppm Ca; d) pH 5, 1.88 x 10^{-1} moles/l Ca; e) pH 7, 1.88 x 10^{-1} moles/l Ca; f) pH 9, 1.88 x 10^{-1} moles/l Ca.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viewed under SEM (Plate 4.10a). Adjusting the pH of the protein slurry to 7 caused a significant change in the fibrosity of the texturized product as compared to samples at pH 5. The protein sheets were more separated, thus appeared spongy, as was evident from the presence of numerous holes or microcavities in the sheets (Plates 4.9b and 4.10b).

Under the more alkaline condition (pH 9), which caused partial solubilization of the protein, the sheets appeared disorganized following the freeze-thaw process (Plates 4.9c, 4.10c). The solubilizing effect of the increased pH resulted in a highly gelled, porous and spongy protein mass, as indicated by the presence of a greater number of holes in the protein sheets (Plate 4.10c).

The protein sheets were comparatively thinner and closer than they appear on the macrostructural level, after freezing at -60°C. A cross-sectional view revealed the sheets in a rosette arrangement, however, a lateral view showed evidence of parallel orientation (Plates 4.11a,b,c). This macrostructural arrangement was not observed in the protein slurry texturized at pH 9 (Plate 4.9c), which might have been masked by the collapse of the weak structure when the sample was sliced for observation. Under the scanning electron microscope, distinct differences of surface topography of the protein sheets can be observed. A coarse surface was exhibited in the sample at pH 5 (Plate 4.11d) compared to the samples at the same temperature and at

Plate 4.11 Macrophotographs and scanning electron micrographs of freeze texturized protein slurry, 0 ppm Ca, -60°C: a) and d) pH 5; b) and e) pH 7; c) and f) pH 9.

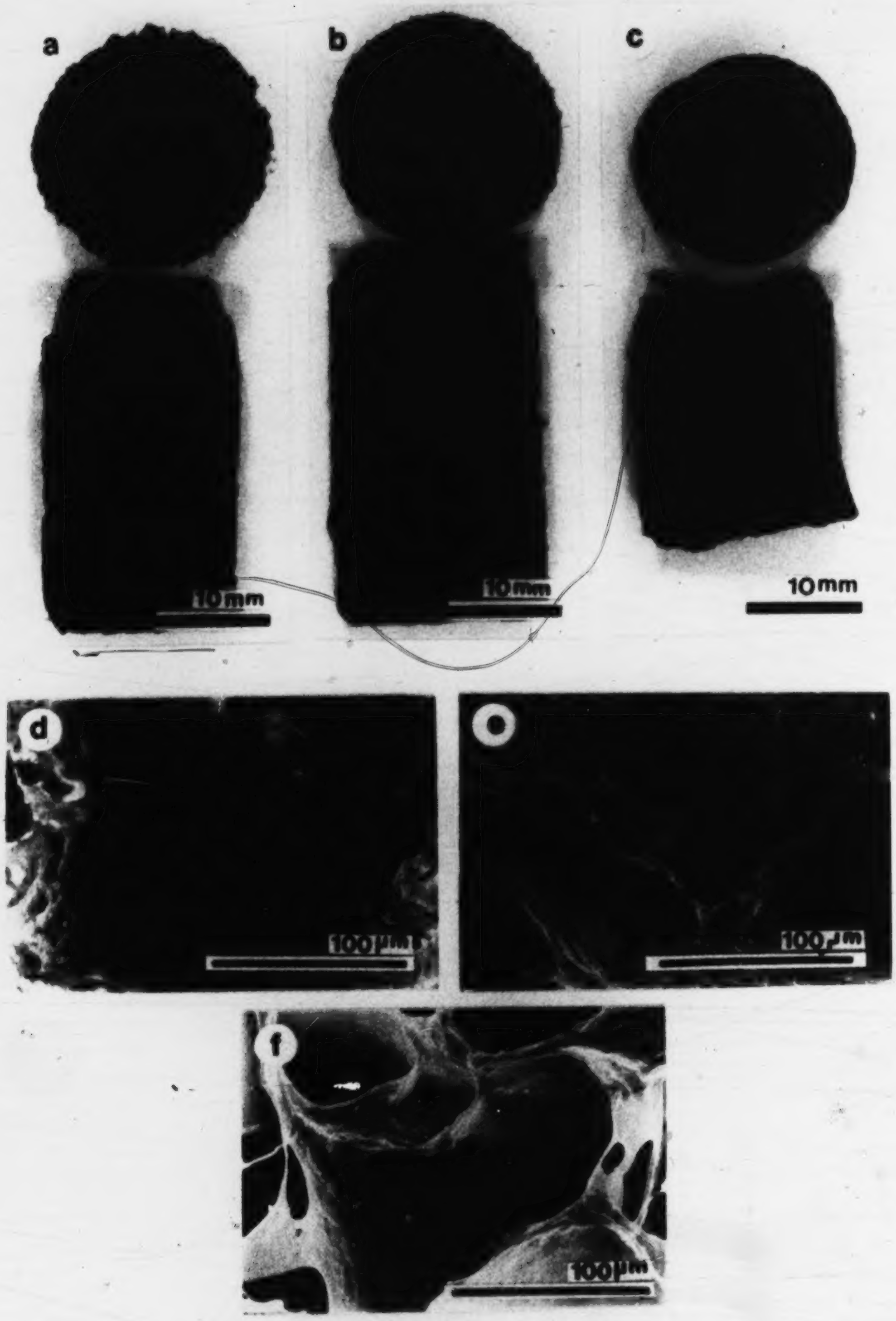
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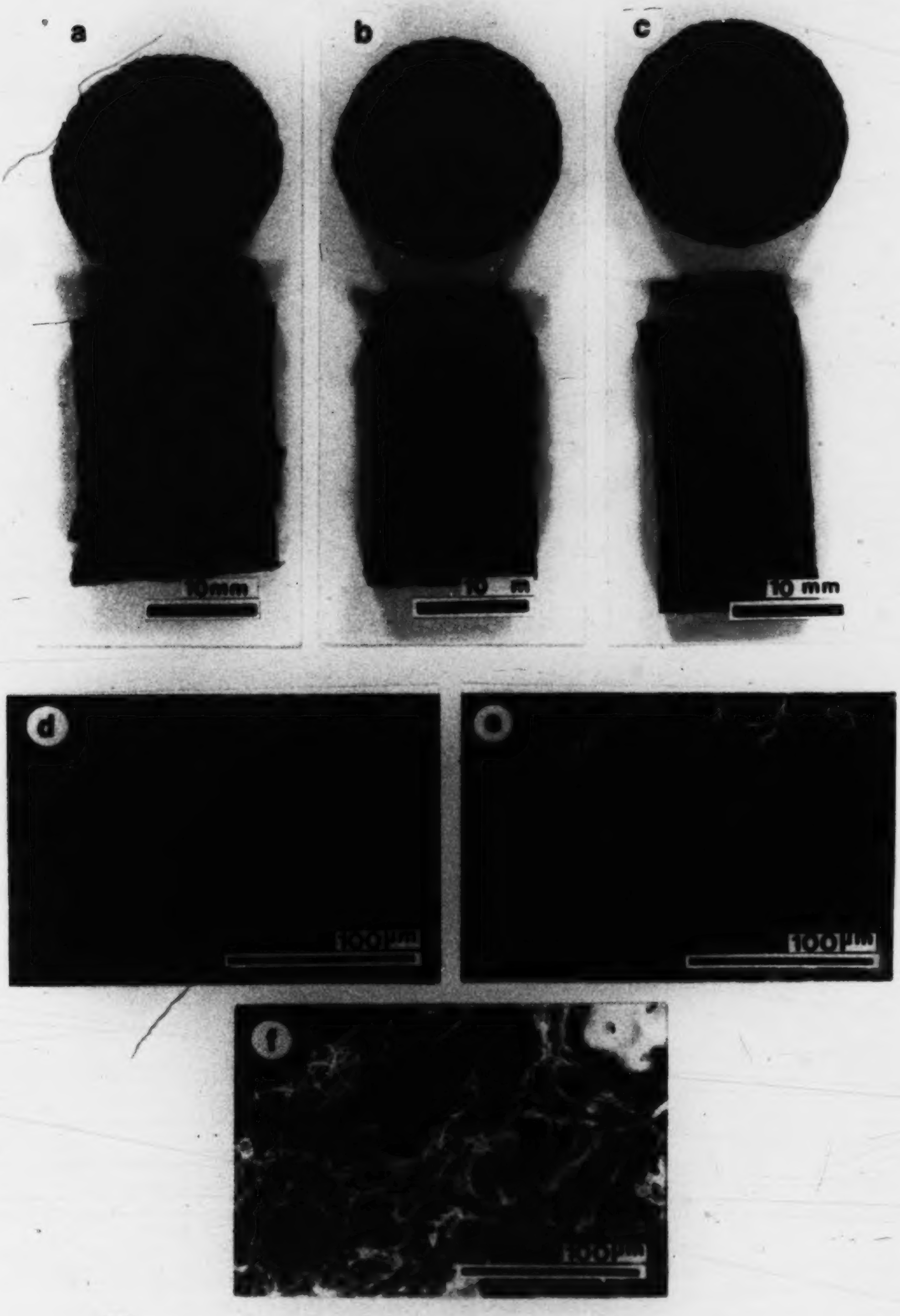
Plate 4.12 Macrophotographs and scanning electron micrographs of freeze texturized protein slurry, 1.88 x 10^{-1} moles/l Ca, -60°C: a) and d) pH 5; b) and e) pH 7; c) and f) pH 9.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-25°C. Such a difference in surface appearance can be attributed to the insufficient mechanical forces exerted by the finer ice crystals at -60°C upon the concentrated protein slurry at pH 5. However, at higher pH's, the solubilizing effect of the alkali had a more pronounced effect in determining the nature of the surface topography of the protein sheets (Plates 4.11e,f). A possible reason could be that proteins at isoelectric point yield a thick slurry which, upon concentration between the elongated ice crystals, could be compressed into relatively regular patterns according to the orientation of the ice crystals. However, solubilized or partially solubilized proteins (e.g. at pH 9) give a thin slurry which becomes resilient toward the mechanical compression of the growing ice crystal. This should explain the relatively large open cells and disorganized protein sheets observed at higher pH values. The final freeze texturized product was fragile and posed handling problems.

Differences in the orientation of the protein sheets due to freezing at -25 and -60°C can be attributed to the increased freezing rate. Rapid freezing prevents migration of water molecules, which are "instantly immobilized" in their sites (Hallett, 1968). Moreso, fast freezing produces numerous ice crystals of limited thickness, which means formation of finer fibers when texturizing protein slurries (Lawrence, 1981). The protein fiber network produced during freezing is, in effect, an imprint or "negative" of the ice crystal structure (Lawrence *et al.*, 1986).

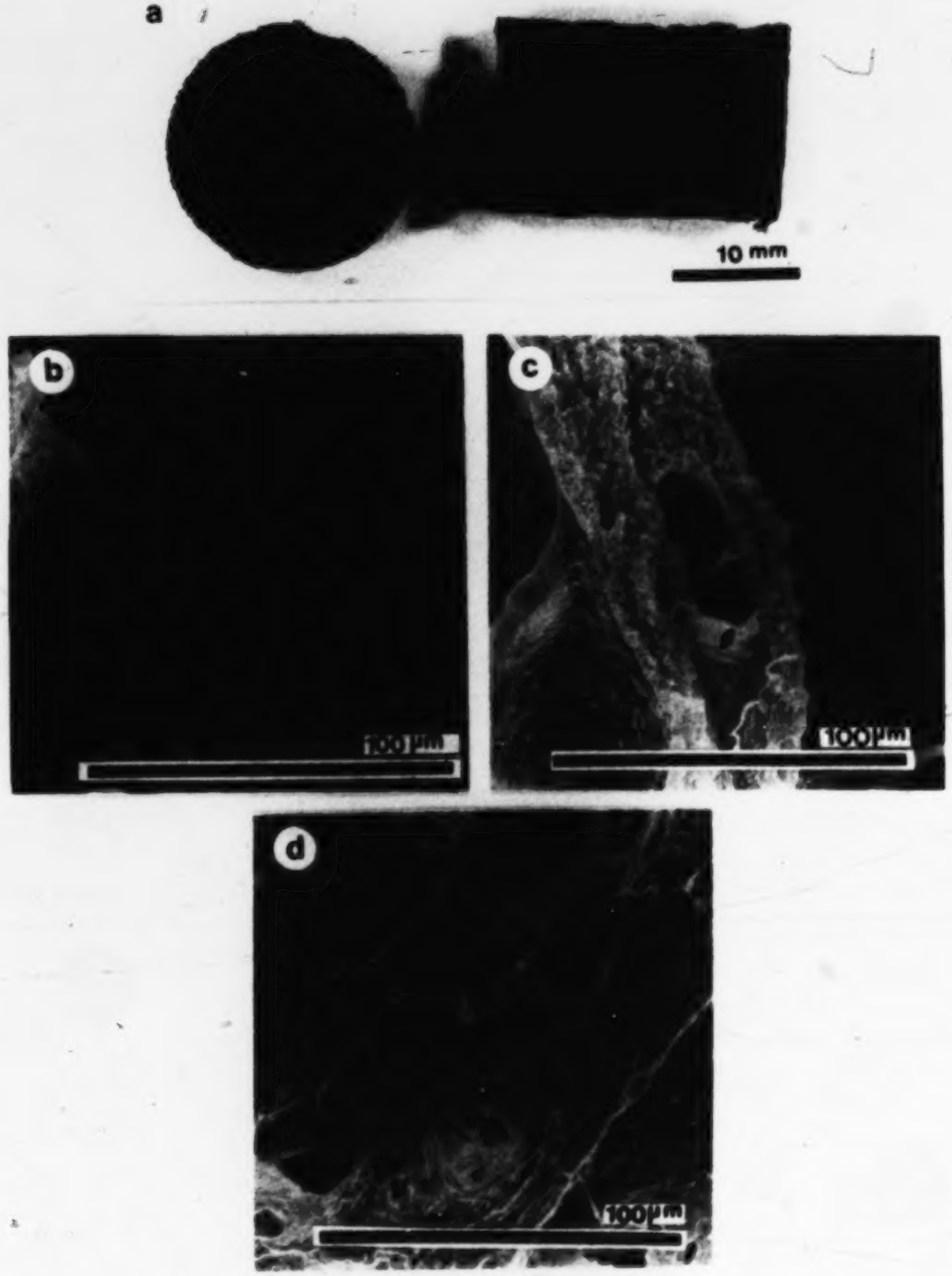
4.4.2.2 Effect of added calcium

The addition of 2.50×10^{-2} moles/l calcium into the protein slurry at pH 5 before freeze texturization at -25°C showed relatively similar fibrosity as the control (pH 5, 0 ppm Ca, -25°C; Plate 4.9a). However, further increase in the amount of calcium added (1.88×10^{-1} moles/l), at the same pH and temperature, caused loss of compactness of the protein sheet structures, as made evident by the formation of bigger spaces between them (Plate 4.9d). This result suggested that the more solubilized the protein becomes due to the increased ionic strength (Figure 4.4), the less resistance is offered to the growth of the ice crystals. Also, a closer examination revealed a decrease in the interconnecting structures between the main protein sheets as the concentration of calcium was increased (Plate 4.10d). However, there is a possibility that, due to the fragility of the protein matrix, these interconnecting structures had been disrupted. Thus, the individual main sheets appeared compressed together as visualized on the macrolevel (Plate 4.9d).

Freezing the protein slurries containing calcium at -60°C yielded the rosette arrangement of sheet structures characteristic at this freezing temperature (Plate 4.12a). The presence of a greater number of finer protein fibers (or interconnecting structures) was observed at higher magnification. Very evident differences in the structure can be seen in the protein slurries freeze texturized at -25 and -60°C (Plates 4.9d,e,f and 4.12a,b,c). At this point, the effect of calcium on structure formation can be clearly demonstrated by comparing the photomicrographs (Plates 4.11 and 4.12). The differences in the surface topography of samples at pH 5 without calcium (Plate 4.11d) and with calcium (Plate 12d) could possibly be attributed to the increased solubilization of the protein. This is due to the increased ionic strength upon addition of 1.88 x 10^{-1} moles/l calcium chloride in the protein system. The effect of calcium was made more evident in the protein slurry at pH's 7 and 9. As indicated in the previous section, the effect of increasing concentrations of calcium were not significantly indicated in terms of the amount of soluble protein in the thawed liquid. However, these effects were clearly demonstrated both in the macro- and microstructures of the texturized protein samples (compare Plates 4.11e,f and 4.12e,f).

Addition of 2.50 x 10^{-3} moles/l calcium into the protein slurry at pH 7 (-25°C) seemed to have no apparent structural difference under the ordinary light photography (compare Plates 4.9b and 4.13a). However, using SEM, the effect could be illustrated (compare Plates 4.13b,c). The surface topography was also different, as indicated by the granularity in the calcium-treated proteins (Plate 4.13c), which may be influenced by aggregation due to the added calcium. Increasing the calcium content (1.88 x 10^{-1} moles/l

Plate 4.13 Macrophotographs and scanning electron micrographs of freeze texturized protein slurry at pH 7, -25°C: a) 2.50×10^{-2} moles/l Ca; b) 0 ppm Ca (SEM); c) 2.50×10^{-2} moles/l Ca (SEM); and d) 1.88×10^{-1} moles/l Ca (SEM).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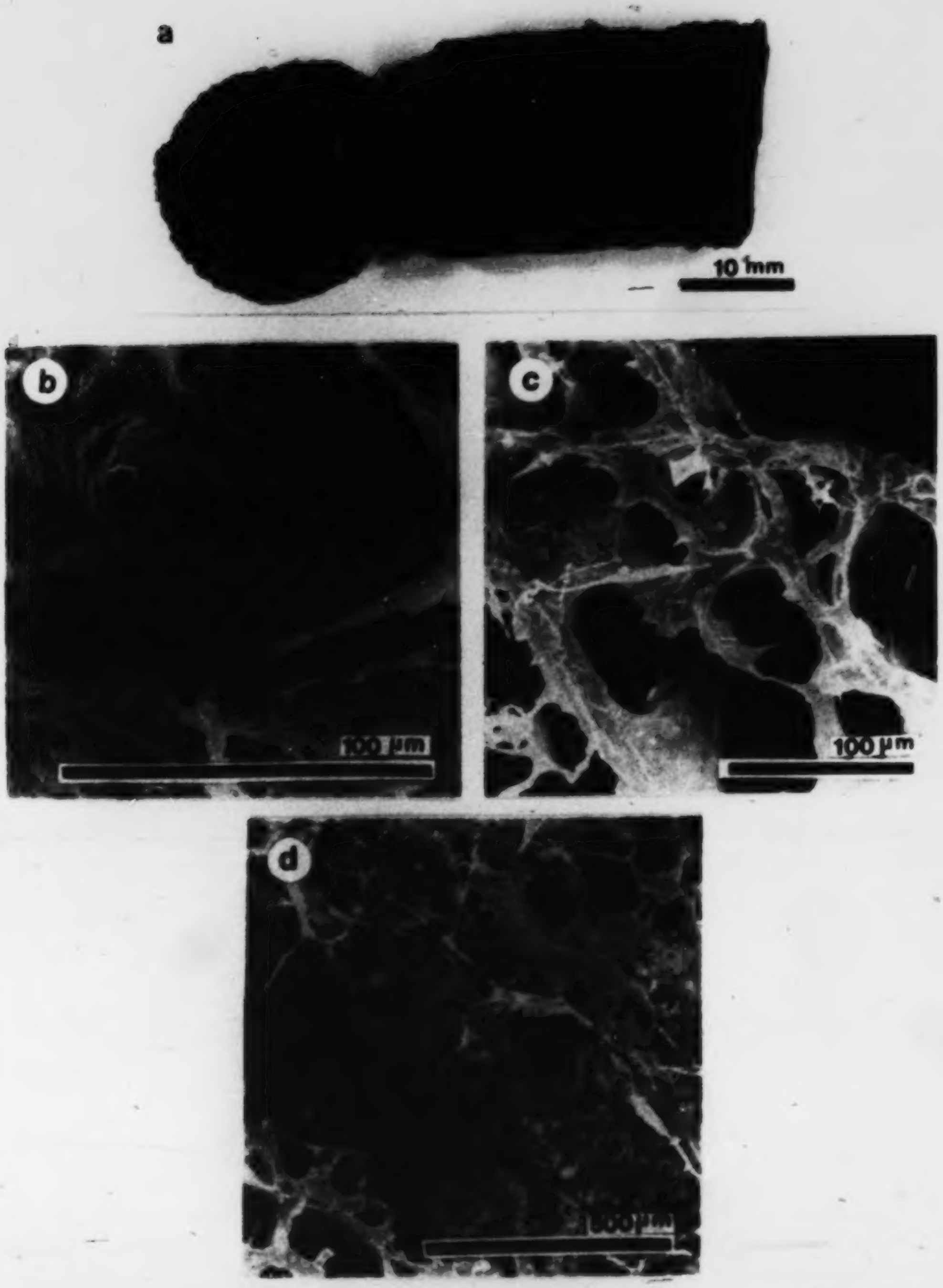
Ca) in the protein system resulted in the formation of numerous holes and microcavities in the sheet structures (Plate 4.13d). The effect of calcium (at pH 7) can also be distinguished by comparing it with texturized protein slurries at pH 5 (Plate 4.10a,d and b,e).

The marked difference in the structural pattern of the texturized protein, due to freezing at -60°C, was exhibited even in the presence of calcium (Plate 4.12b,e). Again, the finer fibers were clearly demonstrated as a result of the rapid freezing.

Increasing the pH to 9 and addition of 2.50 x 10^{-3} moles/l calcium showed a marked change in the structure of the textured protein (compare Plates 4.9c and 4.14a). There was a noticeable reduction in the gelation of the texturized protein after addition of calcium, which could be seen even in the photomicrographs (Plates 4.14b, 4.10c and 4.14c). The sheet structures appeared organized, although numerous microcavities were still present. On the other hand, addition of 1.88 x 10^{-1} moles/l calcium seemed to demonstrate no apparent effect (compare macrographs 4.9f and 4.14a). It was also difficult to ascertain differences in the structure even under the SEM (Plates 4.10f, 4.14d).

Comparison of the structures of the gelled proteins at pH's 7 and 9 without added calcium, and the calcium-treated samples at the same pH's showed clear evidence of the calcium binding effect in the structure formation. This implies that the structure lost under alkaline conditions

Plate 4.14 Macrophotographs and scanning electron micrographs of freeze texturized protein slurry at pH 9, -25°C: a) 2.50×10^{-2} moles/l Ca; b) 0 ppm Ca (SEM); c) and d) 2.50×10^{-2} moles/l Ca (SEM).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could be restored in the presence of calcium ions in the protein system. It is known that peptide chains can form cross-linkages in the presence of polyvalent cations, consequently causing a tightening of the structure (Harowitz, 1950). In the case of rigor and post-rigor muscle, some of the bivalent metals (e.g. Mg, Ca, Zn) are bound to the structural proteins so tightly that it is impossible to remove them completely by treatment with ion exchanger (Hamm, 1960). Calcium binding is also responsible for the structure and texture formation in soybean curd products (Hashizume and Watanabe, 1975; Hashizume *et al.*, 1974). Further, Appu Rao and Narasinga Rao (1975) found insignificant calcium binding of the soy protein (11S) at pH 5.5, but substantial binding at pH 7.8. Significant calcium binding was also noted in leaf protein and gluten at pH 9 (-25°C) as indicated by formation of larger molecular weight fractions (Wallace and Satterlee, 1977). Studies had shown that the imidazole groups of the histidine residues (Appu Rao and Narasinga, 1975) and the negatively-charged acidic amino acid residues (Hashizume *et al.*, 1974) of the protein molecules were the binding sites for the calcium ions.

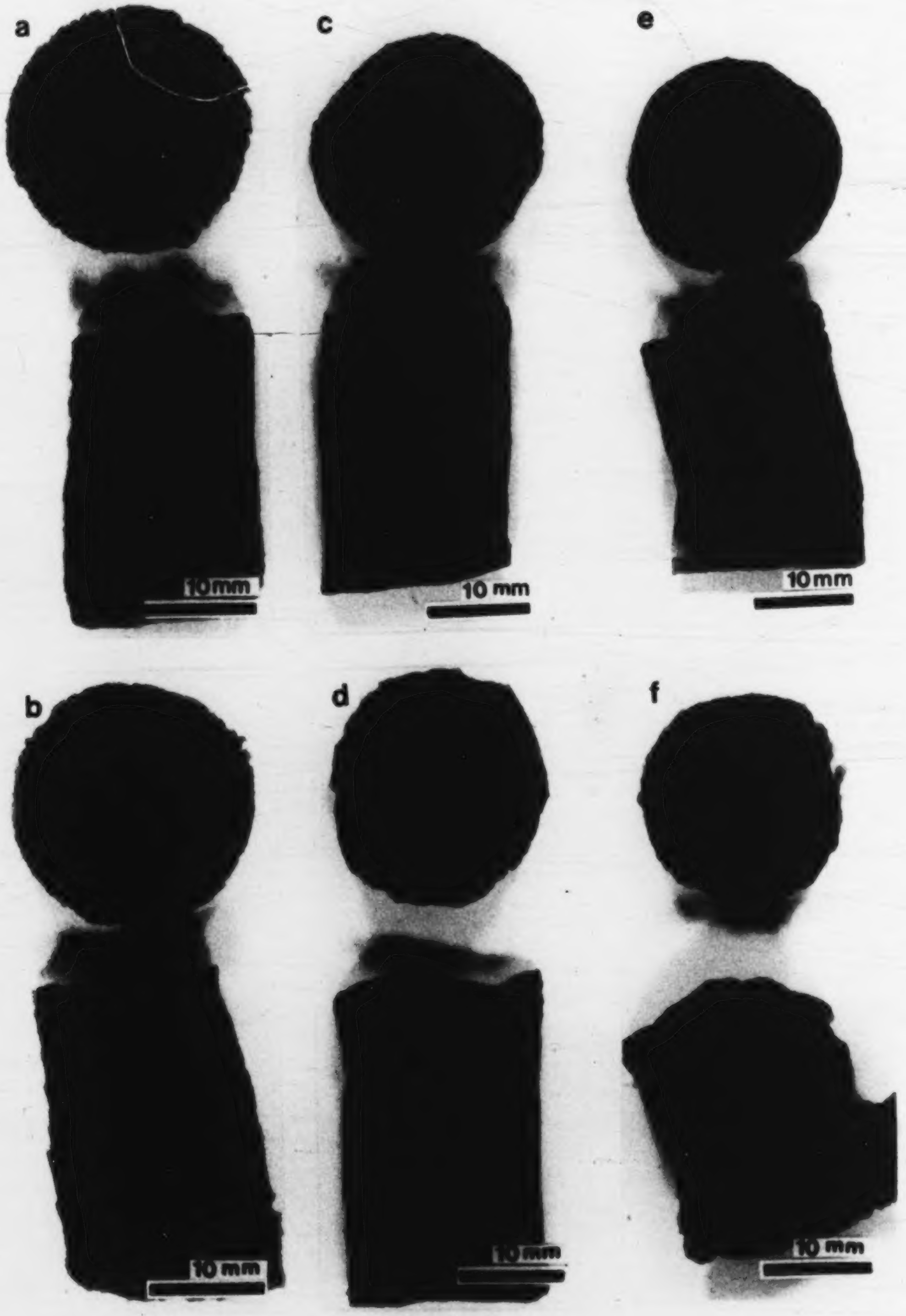
Increasing the pH of the protein slurry from 7 to 9 and freeze texturization at -60°C showed no apparent differences in structure as viewed under ordinary light photography. The more compact texturized protein containing 1.88×10^{-1} moles/l calcium distinguished it from the lower concentration (2.50×10^{-2} moles/l Ca) (Plates 4.12c,f).

4.4.2.3 Effect of added urea

The insignificant difference in protein solubility at pH 5 and in the presence of urea was also reflected in the structure of the texturized protein. There seemed to be no differences in the macro- and microstructres of the products treated with 0.1 moles/l urea at pH 5 (-25°C) (comparing Plates 4.9a vs. 4.15a; 4.10a vs. 4.16a). There were also no apparent differences in the structure of the protein sheets after addition of 0.7 moles/l urea (Plate 4.15b). However, a closer examination of the urea-treated textured protein showed relatively thinner or more flattened main and interconnecting protein sheets (Plate 4.16b). This structural difference could possibly be corralated with the slight increase in solubilized protein in the thawed liquid upon addition of 0.7 moles/l urea (Figure 4.6).

Freezing the protein slurry containing 0.1 moles/l urea at -60°C (pH 5) did not show any marked differences in the fiber structure from the untreated product at the same temperature on the macrolevel. There were just slight differences in the surface topography (granularity) between the control sample and the urea-treated protein. On the other hand, protein slurries containing 0.1 moles/l urea texturized at -25 and -60°C seemed to yield similar structural arrangement (compare micrographs 4.15a and 4.16a). Some peculiarities were also observed in the case of slurries containing 0.7 moles/l urea. At -25°C, the sheets appeared more compact, whereas at -60°C they were more

Plate 4.15 Macrophotographs of freeze texturized protein slurry at -25°C: a) pH 5, 0.1 mole/l urea; b) pH 5, 0.7 mole/l urea; c) pH 7, 0.1 mole/l urea; d) pH 7, 0.7 mole/l urea; e) pH 9, 0.1 mole/l urea; f) pH 9, 0.7 mole/l urea.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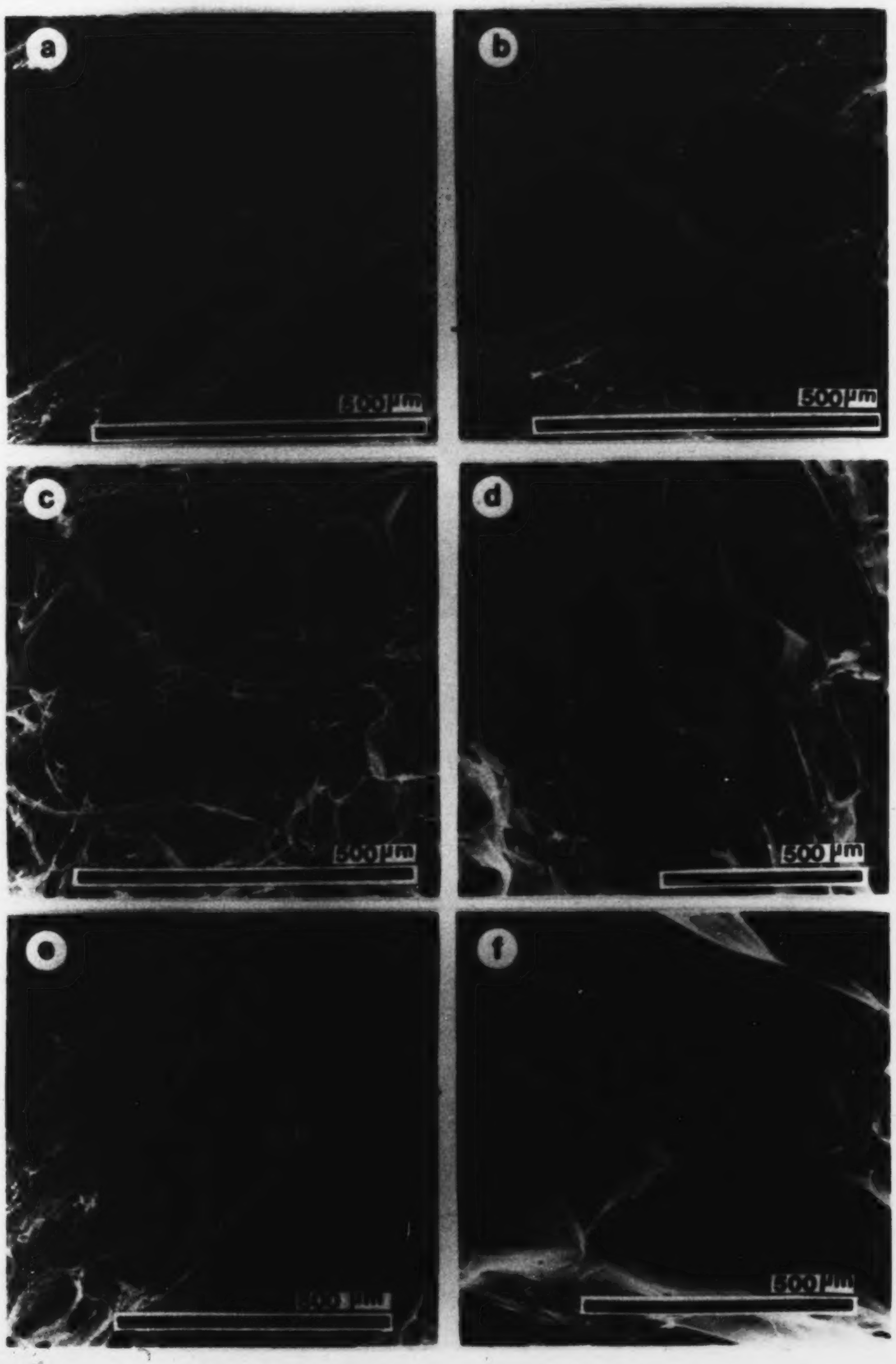
10 mm

Plate 4.16 Scanning electron micrographs of freeze texturized protein slurry at -25°C: a) pH 5, 0.1 mole/l urea; b) pH 5, 0.7 mole/l urea; c) pH 7, 0.1 mole/l urea; d) pH 7, 0.7 mole/l urea; e) pH 9, 0.1 mole/l urea; f) pH 9, 0.7 mole/l urea.


a
500 µm
b
500 µm
c
500 µm
d
500 µm
e
500 µm
f
500 µm

separated. Evidence of the slight solubilizing effect caused by the increased concentration of urea could be demonstrated by comparing the surface topography. Aside from the more flattened sheet structures, the surface appeared relatively smooth at 0.7 mole/l urea added into the slurry.

There were structural differences due to the presence of urea at higher pH values. Addition of 0.1 moles/l urea at pH 7 into the protein slurry and freeze texturization at -25°C, resulted in a gelled product (Plate 4.15c). However, even under these conditions the product appeared organized (Plate 4.16c) compared to the untreated ones under the same processing conditions (Plate 4.10b). It seemed that the presence of urea provided additional resiliency to the protein sheets (Plate 4.16c), maintaining the structure during handling for microscopical analaysis. Samples without urea appeared more fragile (Plate 4.10b). Further addition of urea (0.7 moles/l) solubilized the protein, giving thinner sheet structures (Plate 4.16d). This solubilizing effect can be clearly visualized by comparing the surface topography of the products. A sample containing 0.1 moles/l urea (pH 7) showed granularity (Plate 4.16c), while that containing 0.7 moles/l urea had a smoother surface (Plate 4.16d).

Increases in the degree of porosity and gelation were observed in the protein slurry frozen at -60°C, quite similar to sample texturized at -25°C. Under ordinary light photography, the overall structure appeared as normally observed at this texturizing temperature; however, microscopic analyses revealed distinct differences. Texturized protein containing 0.1 mole/l urea still possessed organization, whereas 0.7 mole/l urea-treated protein seemed to have localized aggregation or fusion. Perhaps this result was due to the failure to achieve homogeneous distribution of the urea in the protein slurry during the preparation stages.

Increasing the pH from 7 to 9 and in the presence of urea resulted in a totally gelled and spongy freeze texturized protein, both at -25 and -60°C (Plates 4.15e,f; 4.16e,f). The macro- and microstructures of the protein sheets differed to a greater extent than any of the other treatments given. The characteristic resiliency of the structures in the presence of urea was enhanced at this alkaline condition. The addition of 0.1 mole/l urea caused gelation and sponginess, however, the structural integrity of the textured protein mass seemed to be retained (Plate 4.16e) compared to the sample subjected to alkaline pH (i.e. pH 9) only (Plate 10c). The smoothness of the surface topography suggested solubilizing effect both of strong alkalinity and urea (compare Plate 4.16c,e). Increasing the concentration of urea to 0.7 moles/l had a tremendous effect on the structure of the protein sheets. The textured product, which actually was converted into a gel (Plate 4.15f), yielded sheet structures that were thin, showing greater flexibility and smooth surface topography (Plate

4.16f). Owing to the elasticity of the protein sheets, the air cell walls were well-preserved, as shown in Plate 4.16f.

At the lower texturizing temperature (-60°C), the effect seemed to be the same as those samples at -25°C (0.7 moles/l urea, pH 9). Similar gelation of the freeze texturized protein was observed, but the rosette organization was totally lost under those conditions. Scanning election microscopy revealed the presence of flexible sheet structure and well-preserved air cells in the protein matrices. Probably, the characteristic microstructure illustrated in the textured product containing low concentrations of urea at alkaline pH's was due to the disrupted hydrogen and hydrophobic bonds in the protein system. It is also possible that the hydrophobic bonds were cleaved due to the increased concentration of urea, together with the proteins, in the unfrozen liquid portion of the freezing system. Urea brings about protein denaturation by breaking interpeptide hydrogen bonds (Kauzmann, 1959). If hydrophobic bonds are weakened at 0°C, and if both hydrogen and hydrophobic bonds are required to maintain the native protein structure, then any weakening of the hydrophobic bonds (such as by lowering the temperature) will make it easier for urea to bring about denaturation (Kauzmann, 1959).

At this point, based on the observed macro- and microstuctures of the freeze texturized protein slurries, it could be stated that insoluble rather than soluble proteins can be texturized. The loss of texturization in the protein slurry at alkaline pH can be partially restored by addition of calcium ions.

4.4.3 Texture Analysis

4.4.3.1 Effect of pH

The protein slurry at pH 5, the isoelectric point of meat proteins, has been characterized by having strong protein-protein interactions via ionic and hydrogen bonding (Hofmann and Hamm, 1978). Adjusting the pH to 7 causes an increase in the number of negative hydroxyl ions in the protein slurry, thereby creating net negative ionic charge surrounding the protein molecules. In the present study, this seemed to have caused weakening of the protein-protein interactions as reflected in the increased porosity and loss of structural integrity of the freeze texturized product. The structural differences brought about by the pH changes were indicated in the significant decrease ($P<0.01$) in the compressive force from 42.50 kg (pH 5) to 24.33 kg (pH 7) (Figure 4.8). Further alterations in the intermolecular interactions occurred when the pH was raised to 9, causing the textural strength of the protein matrix to be reduced to 6.17 kg.

Additional structural differences were brought about by the variation in the freezing temperature as manifested in the significant reduction ($P<0.01$) of the compressive force in protein slurries freeze texturized at -60°C (Figure 4.9).

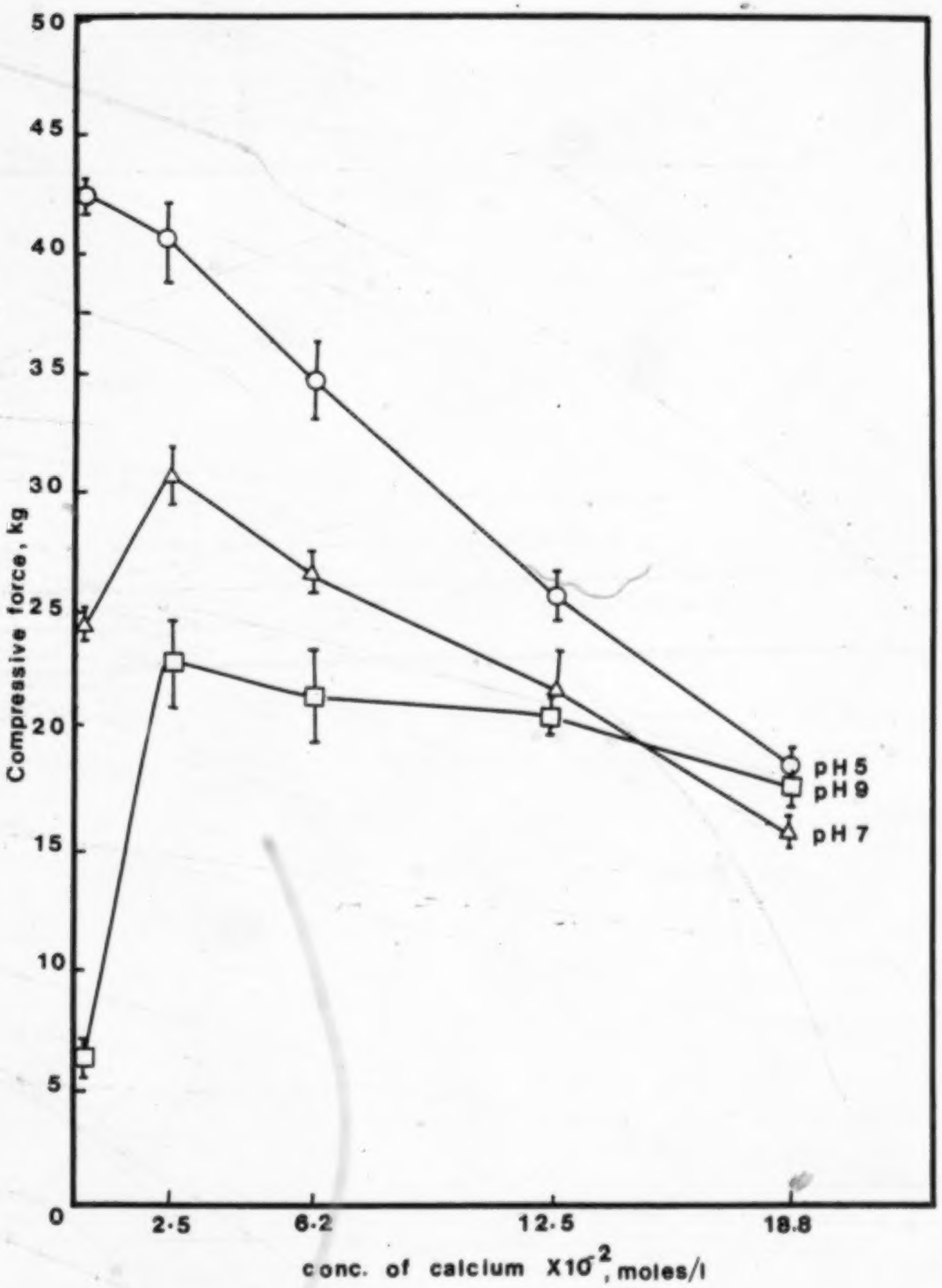


Figure 4.8 Compressive force values of freeze texturized protein slurry at different pH's and concentrations of calcium at -25°C.

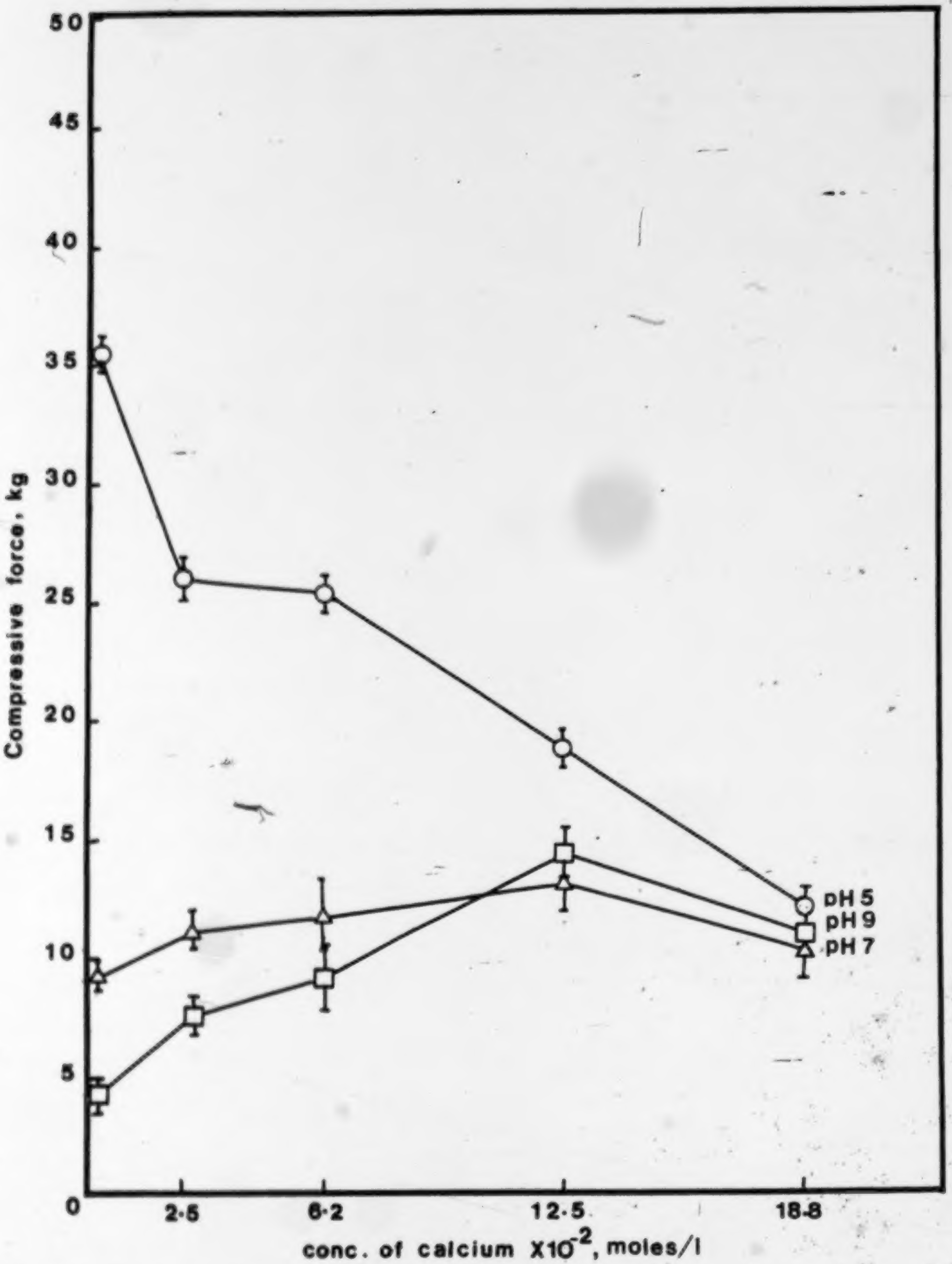


Figure 4.9 Compressive force values of freeze texturized protein slurry at different pH's and concentrations of calcium at -60°C.

This result confirmed that high textural strength is dependent on the presence of thick and more parallel oriented protein sheet structure in the freeze texturized product and insoluble proteins.

4.4.3.2 Effect of added calcium

The addition of calcium up to 1.88 x 10^{-1} moles/l in the protein slurry at pH 5, which had caused a considerable amount of soluble proteins in the thawed liquid, was manifested in the loss of compactness or fibrosity. These effects were reflected in the significant decrease ($P<0.01$) in the compressive force values of the texturized protein (Figure 4.8). The differences both in the structural and textural properties can be attributed to the weakening of the forces stabilizing the protein matrix, possibly because of the increase in the number of positively charged calcium ions in the protein system. The addition of $CaCl_2$ may have a specific Ca^{2+} effect as well as a more general effect due to an increase in ionic strength; this latter effect may lead to greater solubilization of the proteins (Figures 4.4 and 4.5). Although chloride ions were also present (from $CaCl_2$), they may have neutralized the hydrogen ions (H^+) (Fennema, 1977) present in the protein system. Consequently, the final product became loosened and soft, probably because of the disrupted electrostatic forces between the neighbouring molecules.

Quite evident in the samples frozen at -60°C was the relatively higher degree of interconnections (Plate 4.17) compared to products at $-25°C$. However, the compressive force was significantly lower ($P<0.01$) than those product freeze texturized at the latter temperature (compare Figures 4.8 and 4.9, pH 5). This could be the result of the less compression provided by the finer ice crystals on the frozen protein mass.

The addition of 2.50×10^{-3} moles/l calcium into the protein slurry at pH 7 ($-25°C$) showed an increase in the compressive force value (Figure 4.8). Increasing the pH from 5 to 7 increased the net negative ions (OH^-) in the system which could possibly neutralize the hydrogen of the carboxyl-containing amino acid residues. Under this condition, it could be assumed that calcium binding took place between the carboxylate ions in the polypeptide chains, thereby slightly increasing the intermolecular attractions among the protein molecules. However, it should be noted that the introduced calcium binding was not sufficient to compensate for the ionic bonding lost when the latter was disrupted upon adjustment of pH from 5 to 7 because the compressive force of the product (pH 7 plus 2.50×10^{-3} moles/l calcium) was still lower than the control (pH 5, without calcium) (Figure 4.8). Further addition of calcium (up to 1.88×10^{-2} moles/l Ca) resulted in weakening of the matrix structure due to the solubilizing effect of the increased ionic strength in the freezing system. The gelation accompanying this event, consequently, reduced the textural strength of the final product from 24.33 kg to 15.5

kg (Figure 4.8). Freeze texturing the protein slurries at pH 7 (-60°C) in the presence of calcium gave significantly lower compressive force values (Figure 4.9) compared to those products at -25°C (Figure 4.8).

Under more alkaline condition (pH 9), the addition of 2.50×10^{-3} moles/l Ca was manifested by an apparent increase in the compressive force of the textured protein (Figure 4.8). Again, this result suggested binding of calcium to the protein molecules. Further addition of calcium into the slurry showed evidence of quite oriented sheet structures, however, with significantly reduced textural strength (Figure 4.8). The compressive force of the texturized protein containing 1.88×10^{-1} moles/l Ca was still significantly higher than the sample without calcium. This result suggested two opposing forces present in the protein system under these conditions, namely attraction due to calcium binding, and repulsion due to increased hydroxyl ions. The calcium bridging occurred at specific and limited binding sites in the protein molecules.

The increase in the compressive force due to the added calcium and freeze texturization at -60°C is shown in Figure 4.9. Again, the strength of the protein at this temperature, in terms of textural strength, was found to be significantly lower than at -25°C.

4.4.3.3 Effect of added urea

The minimum increase in amount of soluble protein in the presence of increasing amount of urea, although insignificant, showed quite different textural response. These minimum increases in soluble proteins were demonstrated in significant reduction in compressive force values of protein textured at pH 5 (-25°C) (Figure 4.10). Significant differences in textural strength were also observed at -60°C (Figure 4.11).

Statistical analysis showed that there were highly significant differences ($P<0.01$) in textural strength due to pH, concentration of urea and freezing temperature and the interactions of these variables (Figures 4.10 and 4.11). Adjusting the pH from 5 to 9 resulted in gel formation, which consequently reduced the compressive force values. This could be attributed to the weakening of the structure caused by the combined effects of increased alkalinity and presence of urea in the protein system. The protein solubilizing effect of urea is increased under alkaline environments (see Section 4.4.6) (Lapanje, 1978). The results also had indicated that any changes in the chemical composition (or properties) of the raw material, no matter how small or insignificant, would be manifested in the microstructural and textural properties of the final product. For example, the small increments in solubilized protein at increased amounts of urea in the protein slurry showed distinct changes in the structural organization of the protein sheets, which in turn gave significantly lower compressive force values. Furthermore, the combined effects of alkalinity and urea yielded textured protein having

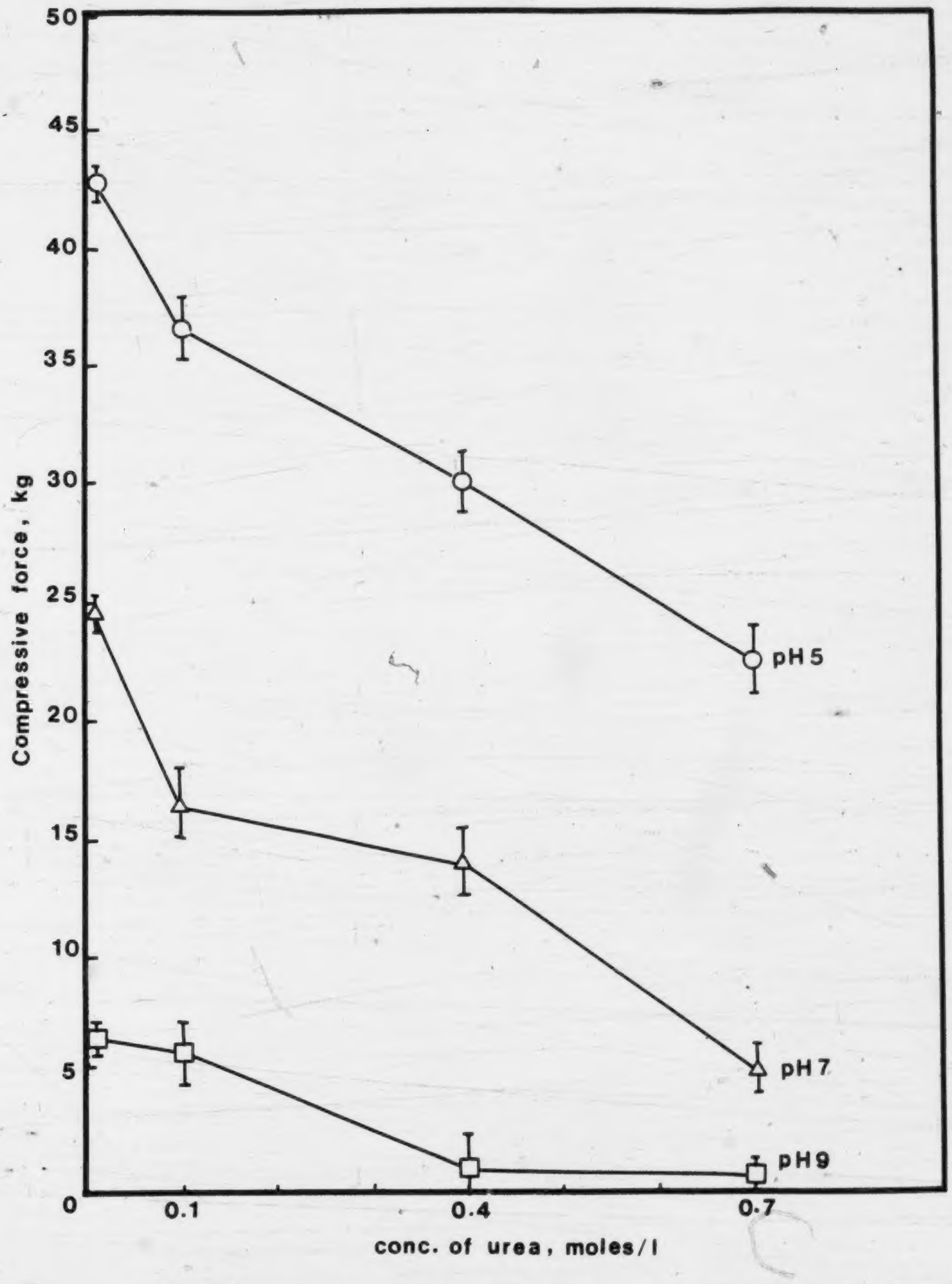


Figure 4.10 Compressive force values of freeze texturized protein slurry at different pH's and concentrations of urea at -25°C.

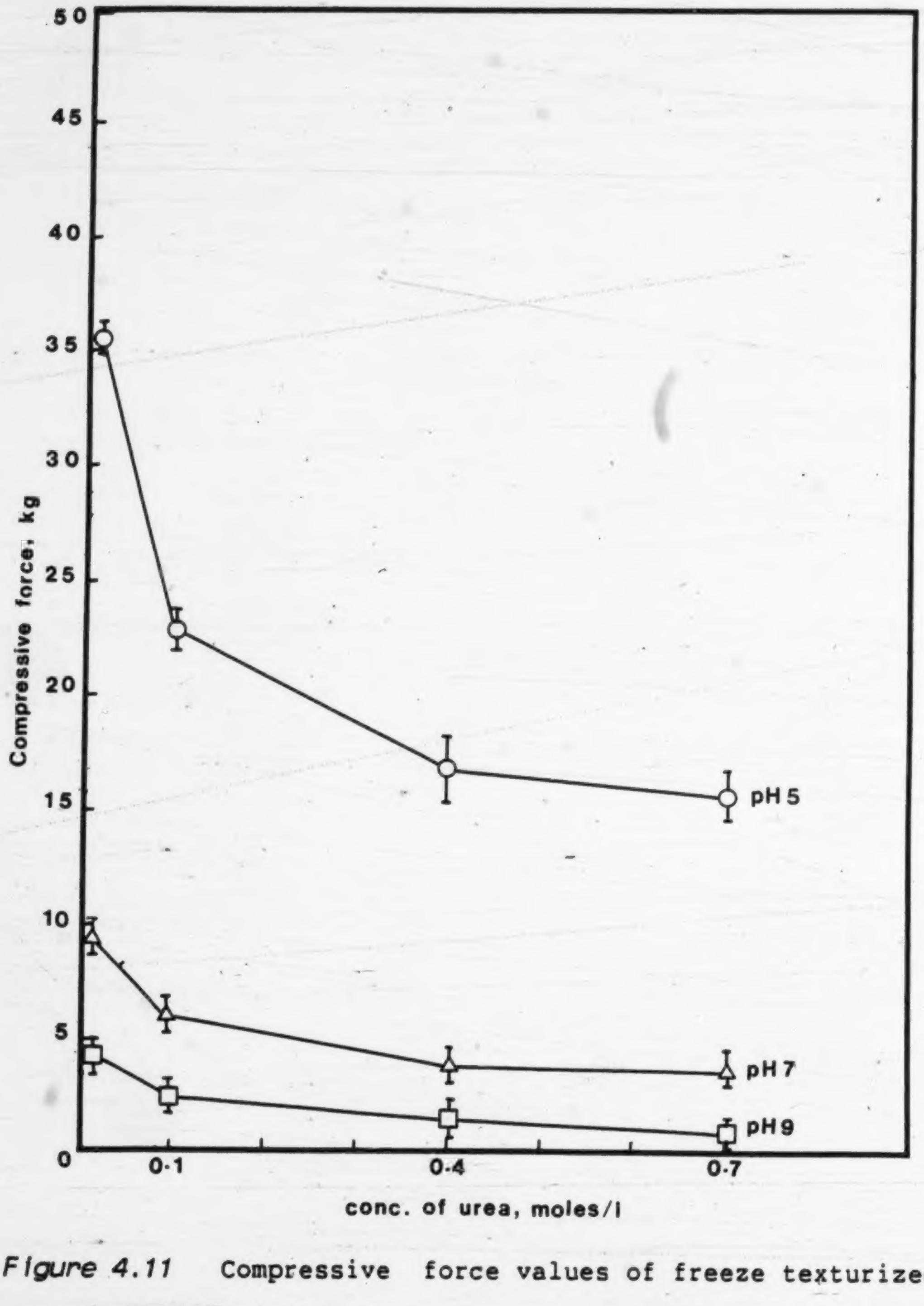


Figure 4.11 Compressive force values of freeze texturized protein slurry at different pH's and concentrations of urea at -60°C.

spongy and thin-sheeted structures, which drastically reduced the textural strength, notably in protein slurries at pH 9 (Figure 4.10). Similar trends were also observed when the protein slurries were freeze texturized at -60°C. Microstructural differences due to freezing temperatures were in turn reflected in significant differences in textural strengths (Figure 4.11).

4.5 Texturization of Whey Proteins

In this study, an attempt was made to freeze texturize protein preparations obtained from cottage cheese whey. The objective was to explore the feasibility of texturizing whey proteins by considering their states, denatured and undenatured.

The main components of the whey protein fraction (β-lactoglobulin and α-lactalbumin) are globular in nature and therefore should be unfolded in order to satisfy the primary requirement for texturization. Thus, the reactive groups in the polypeptide chain are exposed, favoring protein polymerization. Three methods of recovering proteins from cottage cheese whey were employed so that the proteins could be studied at different states. Ultrafiltration (Lewis, 1982) and complexing with polyphosphates (Hartman and Swanson, 1966) method, based on these authors' results, should produce native or undenatured proteins. On the other hand, acid-heat precipitation technique should yield denatured proteins, the heat treatment being sufficient to cause unfolding of the globular whey proteins (Evans and Gordon, 1980). The protein and moisture contents of the whey protein concentrates obtained by these methods are given in Table 4.8. All freeze-dried whey protein preparations were rehydrated to about 10% protein prior to the freeze texturization studies.

Results showed that, after freeze-texturization, there was no fiber or sheet formation, even when the proteins were subjected to various pH treatments. The protein (pH 5) from the ultrafiltration method merely aggregated into small granular particles (Plate 4.17a). Adjusting the pH of the whey protein from the same preparation to 7 and 9 dissolved the protein, which, after the freeze-thaw process, just became turbid, with minimum flocculation.

Fiberization and texturization were also not observed from undenatured whey protein prepared by precipitation with sodium hexametaphosphate at pH 2.5. Similarly, acid-heat denatured whey protein adjusted to pH 5 (from about pH 6) showed no change in appearance after the freeze-thaw process (Plate 4.17). However, adjusting the pH to 7 and 9 showed some cohesiveness upon freezing and thawing. The more alkaline product totally disintegrated during an attempt to heat-set the structure (Plate 4.18a,b). Alkali solubilization of the heat-coagulated protein by heating at 70°C at pH 11.2 (Jelen and Schmidt, 1976) was followed by re-adjusting of the pH to 5, 7 and 9. After freeze texturization, all the products showed a higher degree of cohesiveness, however,

Table 4.8 Protein and moisture contents of freeze dried whey protein concentrates[1].

Method of Preparation	% Protein	% Moisture
Ultrafiltration	44.83 ± 1.67	3.85 ± 0.08
Hexametaphosphate complexing	70.94 ± 2.34	3.30 ± 0.02
Heat-acid precipitation	45.31 ± 2.10	3.35 ± 0.05

[1] Average values of three replicates.

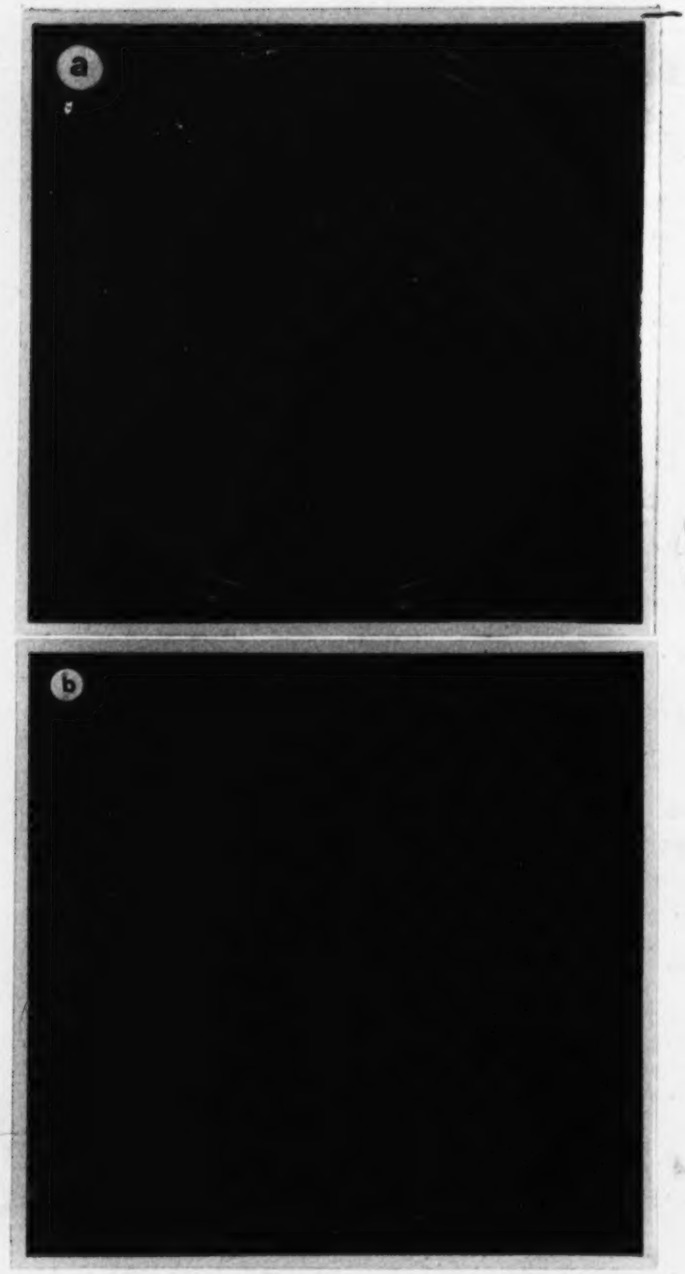


Plate 4.17 Ultrafiltered (a) and acid-heat precipitated (b) whey protein concentrate (pH 5) after the freeze-thaw process.

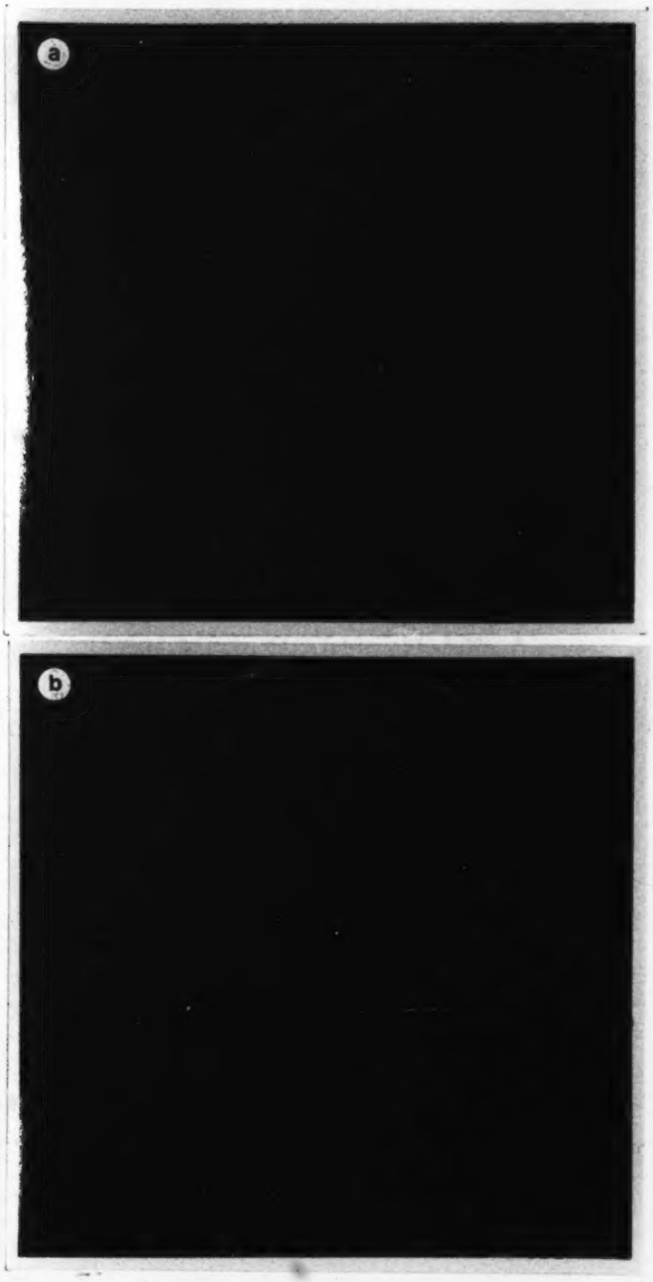


Plate 4.18 Acid-heat precipitated whey protein concentrate, pH 9: a) after the freeze-thaw process; and b) after heat-setting.

the structure disintegrated upon handling. The whey proteins at pH 7 and 9 were more gelled than without the alkali solubilization treatment (Plate 4.19a,b). The behaviour of the various whey protein preparations during the freeze texturization process is summarized in Table 4.9.

The failure of the native globular whey proteins, prepared by ultrafiltration and polyphosphate complexing methods, to texturize supported the findings of various workers that unfolding of the protein molecules is necessary in order to produce fiberization (Huang and Rha, 1974; Pressey and Kelley, 1966). In the solubilized ultrafiltered whey protein (pH 7 and 9), the absence of texturization confirmed the prediction of Lillford (1983) which also supports the findings obtained in the other parts of the study (Section 4.5) that the proteins must be insoluble.

The heat treatment for protein recovery seemed to have no significant effect on the subsequent freeze texturization of the whey proteins. However, alkali solubilization of the heat-precipitated proteins followed by readjustment of the pH to 7 and 9 seemed to have altered the protein molecules. There was an increased cohesiveness in the proteins mass, although insufficient to produce fibrous and cohesive texture.

In the fiber spinning technique, Lundgren (1949) stressed that, if the unfolding protein chains become in contact with the precipitating bath, they are exposed to conditions that favor their interaction. If the chains were

Table 4.9 Response of various whey protein preparations to freeze texturization.

Method of Preparation	Effect of Freeze-texturization	Reference
Ultrafiltration	Nil	Bernal and Jelen (1985)
Hexametaphosphate complexing	Nil	Hartman and Swanson (1966)
Heat-acid precipitation	Some freeze-alignment, no cohesiveness	Jelen (1975)
Alkali solubilization of heat-acid precipitate	Some freeze-alignment, little cohesiveness	Jelen and Schmidt (1976)

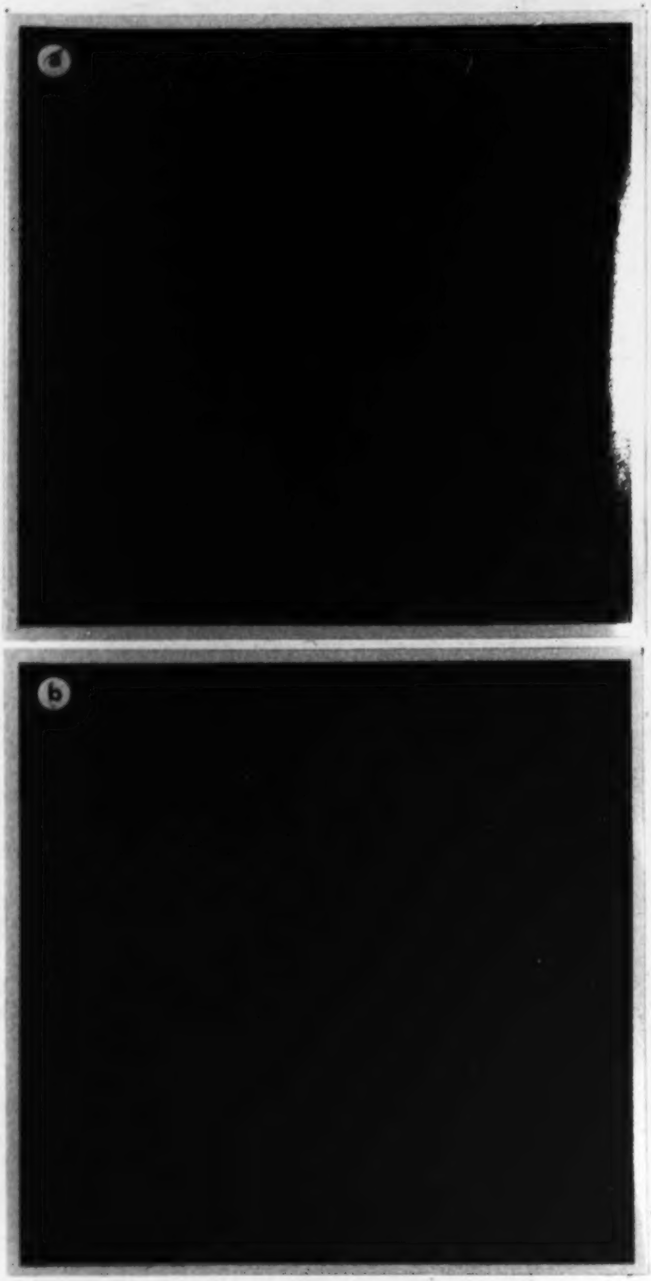


Plate 4.19 Alkali-solubilized, acid-heat precipitated whey protein concentrate, pH 5: a) unfrozen; and b) after the freeze-thaw process.

not unfolded at this stage, their poor coherence as the result of interaction would yield a flocculent instead of fibrillar precipitate. A good example given is the protein slurry derived from poultry meat residues, where the myofibrillar proteins and their extended cross-linking provided a good coherence, even prior to the freezing process. Perhaps, to some extent, the unextended state of whey proteins used in the present study would explain their failure to freeze texturize. Also, the relatively low molecular weights of major whey proteins (β-lactoglobulin, 18,300 daltons; α-lactalbumin, 14,200 daltons) compared to poultry meat residue proteins (see Table 4.3) might be a critical factor to consider in freeze texturizing the whey proteins (R. Jost, Nestlé Switzerland, Personal Communication).

4.6 Proposed Mechanism of Texture Formation upon Freezing

A clear picture of the events taking place during the various chemical treatments given the protein slurry could be visualized by considering the diagram shown in Figure 4.12. Initially, at the isoelectric point the protein strands are held together mainly by ionic or electrostatic bonds. These are represented by the cross-links in the structure (designated by X in Figure 4.12B,C), which were also demonstrated in the scanning electron micrographs of unfrozen protein isolate. Increasing the pH towards alkalinity increases the negative ionic species in the

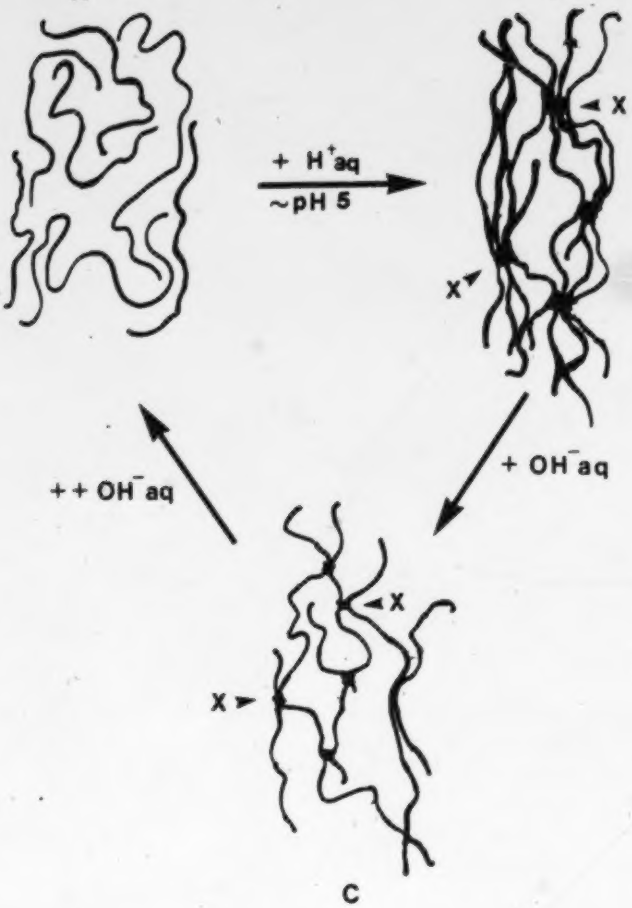


Figure 4.12 Proposed mechanism on the effect of cross-linking on texture formation during freeze texturization (taken in part from Hamm, 1975). (A) randomly oriented protein chains (e.g. protein solution); (B) maximum cross-linking of protein chains (e.g. protein precipitate); and (C) partial cross-linking of protein chains (e.g. partially solubilized protein). X indicates sites of cross-linking.

system so that the protein molecules experience repulsion, thereby decreasing the number of cross-links in the protein matrix (Figure 4.12C). Further addition of the negative hydroxyl ions or any antagonistic species would totally disrupt the cross-linked protein chains due to the magnitude of repulsion among the chains. Such is the case in a protein solution where the protein chains are dispersed completely separated (Figure 4.12A). In the actual experiment, the alkali was added to just cause partial protein solubilization so that cross-linking still existed. The same event takes place upon addition of a limited amount of urea, especially under the alkaline environment, resulting in the loss of texture. However, the loss of texture under alkaline condition could be partially regained in the presence of calcium ions in the protein system. Although calcium binding is possible, the number of binding sites limit its contribution to the formation of cross-links. This was made evident by the lower compressive force values of calcium-treated, freeze texturized protein compared to the untreated, isoelectric protein isolate. This means that the contribution of calcium bridges at alkaline pH was insufficient to match the strong forces provided by the enormous ionic or electrostatic forces present in the protein system at isoelectric point. On the other hand, the possible increase in the concentration of urea in the unfrozen liquid part, coupled with alkaline pH, would be responsible for cleaving the existing hydrogen and hydrophobic linkages.

The disruption of the forces in the protein matrix was demonstrated by the low compressive force values, which in turn were correlated with electron microscopy observations showing the separation of the protein sheet structures or even formation of gelled protein matrices. The weakened forces holding the proteins were reflected in the increased amount of soluble protein in the thawed liquid, obtained following freeze texturization. At this point, it should be mentioned that, during freezing, all the ionic species, proteins and other solutes are concentrated in the unfrozen liquid portion between the parallel oriented ice crystals. The presence of antagonistic species around the protein molecules would limit or prevent molecular interactions. Thus, the protein slurry would partially texturize or fail to do so. Also, the loosely-bound protein molecules would easily solubilize in the melted ice upon thawing the freeze texturized protein.

Additional information on the mechanism of texturization has been obtained by the modification of the composition of the poultry meat protein using the freeze texturization technique. Based on the results gathered in this study, it could be concluded that most, if not all, of the intermolecular cross-links that form between the myofibrillar proteins present in the slurry are probably secondary in character. This is so since they are capable of being disrupted by treatment by alkali and in the presence of urea. Under isoelectric pH, the forces holding the protein molecules could be disrupted also by additon of excess amounts of calcium ions. The cleavage due to these chemical forces will solubilize the proteins, but will not freeze texturize. Therefore, it could be stated that these secondary forces (ionic, hydrogen and hydrophobic bonds) are responsible for the texture formation in freeze texturized poultry meat protein.

On the other hand, the inability of the whey proteins to freeze texturize would reflect some inherent physicochemical properties affecting their capabilities to undergo intermolecular polymerization. The latter utilizes the ability of the protein to form junctions (cross-links) of its own molecules to themselves, for example, in fiber formation. Intermolecular polymerization is influenced by the innate characteristics of the proteins, including amino acid composition, size, conformation, and bonds and forces (Pour-El, 1981).

5. SUMMARY AND CONCLUSIONS

The results of this study provide valuable information leading to further understanding of the freeze texturization mechanism in food proteins. The major findings, summarized in the following paragraphs, indicated that freeze texturization is no longer a "state of the art" process but indeed has a strong scientific basis for the observed phenomena.

5.1 Summary of Research Findings

1.

The SDS-PAGE technique had indicated that alkali-extracted and acid-precipitated chicken meat protein slurry contained a wide range of polypeptides of high molecular weight (18,500-248,000 daltons), a critical factor in any protein texturization technique.

2.

Non-directional freezing mode was characterized by the presence of parallel sheet structures intersecting at the geometrical center of the sample, forming a junction. The presence of fused or homogeneous protein mass and the absence of parallel aligned structure in this junction might have caused the lower textural strength of the freeze texturized protein. Coupled with rapid freezing (-196°C), non-directional freezing gave a highly porous product. Indeed, the results suggested that unidirectional freezing mode could achieve formation of more parallel aligned sheet structures, under appropriate freezing temperature (e.g. -25°C). At this freezing temperature ample time was provided to form elongated ice crystals in the freezing system.

3.

In general, as the freezing temperature was decreased the rosette pattern characteristic formed and the product became less compact and more spongy, with low-textural strength. More pronounced parallel oriented protein sheet structures were formed at -25 rather than at -60°C. Results obtained by the microscopic analyses provided additional evidence that rapid freezing results in the formation of numerous small ice crystals and gives the appearance of less fibrosity, while slow cooling yields a more distinct fibrosity after heat setting.

4.

Electron microscopic analysis revealed the occurrence of fiberization and extended protein cross-linking in the freshly alkali-extracted, acid-precipitated protein slurry. Fiber formation at the preparative stages of the process was, in fact, substantiated by the failure of the mechanically deboned poultry meat paste to texturize compared to the protein extracted from this raw material. From these results, it could be concluded that protein concentration (by isoelectric precipitation) is needed to allow intermolecular interactions prior to the freezing process. However, this does not deny the fact that freezing also causes protein concentration and enhances cross-linking in order to establish the structure of the texturized product. The change of organization level from the amorphous protein paste (before freezing) into a parallel aligned protein mass (after freezing) strongly supports the occurrence of further protein-protein interactions during freezing and storage. It could be possible also that the presence of interfering substances, such as high fat content, would prevent protein-protein interactions. Thus, the use of protein isolates would be more appropriate for this process.

5.

The nature of cross-linking of proteins at the different stages of the texturization process was determined by employing protein solubility tests in various aqueous solvents. High solubilities in KOH, KCl or SDS solutions implied that aggregation of the protein molecules in the fresh isolate was predominantly due to ionic bonding. The significant decrease in protein solubility after heat-setting the freeze texturized protein was indicative of the stabilization of the various chemical forces (ionic, hydrogen and hydrophobic bonds) that maintain the structure of the textured product.

6.

The pH of the protein slurry was found to have a great influence both on the macrostructure and texture of the product. The results had pointed out the need to texturize isoelectric protein (pH 5), which is characterized by strong protein-protein interactions via the numerous electrostatic forces. The protein, in the form of slurry, could be compressed between the parallel ice crystals, giving a firm structure. Since freezing causes concentration of proteins and solutes, results strongly suggested that this event may have caused partial protein solubilization, thereby promoting protein interactions which provide the cohesiveness needed to maintain the structural integrity of the textured product. The results had also indicated that, if the proteins were partially solubilized (e.g. at pH 7 or 9) prior to freezing, the final product would be gelled, soft with open structure. Textured protein at pH 5 showed more organized parallel orientation of the sheet structures, possessing higher textural strength than products at pH 7 or 9. From these results, it could be stated that the optimum substrate for freeze texturization seemed to be largely insoluble material in which some protein is dissolved or dissolves local~~ly~~ during freeze concentration. The presence of the solubilized protein on particle surfaces, while not greatly hindering ice crystal growth, should produce an adhesive which sets on heating.

7.

The results obtained in this study had indicated a pronounced effect both of the structural and textural properties of the freeze texturized protein brought about by the combined effects of the increased pH and concentration of calcium or urea. The microstructure of the gelled protein slurry was characterized by the formation of big, thinwalled open cells. A very peculiar resiliency of the sheet structures due to urea treatment was distinguishable from the effect of high calcium content at alkaline pH. Based on the results, there was strong indication of the prominent roles of the ionic bond, dirsupted under alkaline pH and high calcium content, and possibly hydrogen bond, disrupted in the presence of a low concentration of urea at alkaline pH, in providing the structure of the freeze texturized protein. Although protein polymerization through calcium binding was possible under alkaline conditions, the effect was not comparable in its absence and at pH 5.

8.

The changes in the chemical composition of the protein slurry could be manifested in the microstructure, which in turn could be measured in terms of textural strength. The Instron texture measuring assembly was used to apply the longitudinal force and to examine the textual strength, expressed as compressive force, of the freeze textured protein. The results had indicated that, as the protein slurry becomes more alkaline, the net negative charges around the protein molecules cause repulsion, hence, protein solubilization occurs. The weakening of the forces in the protein matrix leads to disorganization of the microstructure. Results showed that this transformation in the structural organization of the protein matrix was responsible for the significant responses of the texture measuring devices.

9.

The failure of the whey protein (undenatured or denatured) to freeze texturize, even after being subjected to various treatments, implied that the concentration of the proteins and other solutes between the elongated ice crystals is just a secondary step in the texturization process. This is additional supporting evidence of the need to achieve conformational changes in the protein molecules before freeze texturization in order to promote and maximize protein-protein interactions; this would be considered the primary step in the process. This statement could be supported by considering the success in texturing alkali-treated poultry meat residue proteins and the failure of the mechanically deboned meat paste to texturize. At this point, even though whey proteins may appear to possess high molecular weights (14,146-1 million daltons; Morr, 1978), only the lower ones are recovered following the traditional methods. Therefore, assuming that the pretreatments or the freezing process itself may have unfolded the proteins, their relatively small molecular sizes could have limited the occurrence of a higher degree of protein-protein interactions. Further, the quality of the amino acid residues (and their states) are also of considerable importance in this respect.

10.

Finally, the freeze texturized, mechanically deboned meat residue protein is only comparable to the original poultry meat in terms of the parallel orientation of the protein mass (sheet) on the macrostructural level. Under the microscopic state, the protein fibers (or, better, sheets) appeared randomly oriented, which was not surprising based on the nature of the freezing process. The compressive force provided by the growing elongated ice crystals seemed not enough to stretch the protein fibers, if the high degree of parallel alignment and stretching (400%) of fibers attained in the fiber spinning process is to be considered (Boyer, 1954). The applied stress leads to orientation in the fibers, which means uniformity of alignment of the chains and organized regions with respect to a specific direction in the fiber, usually the fiber axis (Lundgren, 1949). Based on the results obtained in the present study, there are two levels of orientation exhibited by the freeze texturized protein, namely the distinctly parallel aligned protein sheets and the randomly arranged and totally entangled fibers compressed in these protein sheets. This product characteristic differentiates the freeze texturization process from the other methods of protein texturization.

11.

Overall, the success with the freeze texturization of poultry meat proteins (fibrous type) and the failure of the whey proteins (globular type) to form sheet structures illustrate some important aspects of the freeze texturization process (Figure 4.13). There is a strong indication that protein polymerization has to be initiated

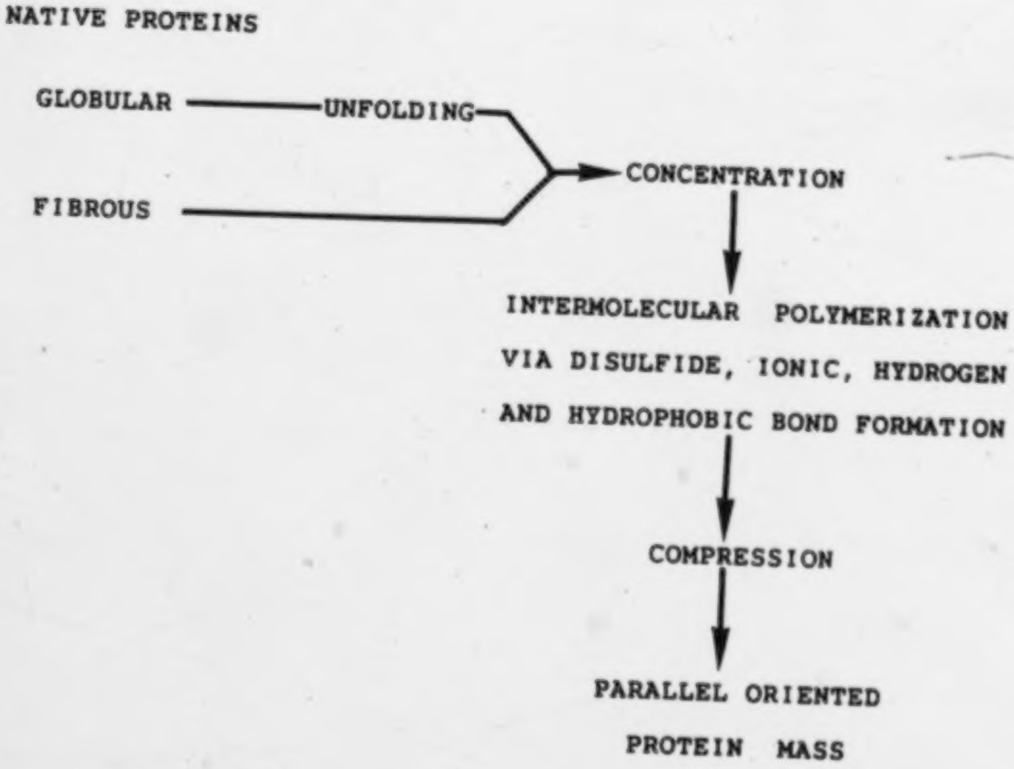

NATIVE PROTEINS
GLOBULAR
UNFOLDING
FIBROUS
CONCENTRATION
INTERMOLECULAR POLYMERIZATION
VIA DISULFIDE, IONIC, HYDROGEN
AND HYDROPHOBIC BOND FORMATION
COMPRESSION
PARALLEL ORIENTED
PROTEIN MASS

Figure 5.1 Key processes in freeze texturization of food proteins.

during the protein preparation following unfolding of the protein molecules. The latter process is required if starting with globular-type proteins. Protein polymerization occurs through ionic, hydrogen, hydrophobic and probably disulfide bond formation. The alkali-extraction process for mechanically deboned poultry meat residues may play an important role in this respect.

It also appeared and could be emphasized that, in the absence of a high degree of protein-protein interactions prior to the freezing step, the proteins will not texturize even if they are concentrated and compressed during the freeze texturization process.

5.2 Recommendations for Future Work

Future studies toward greater understanding of the freeze texturization mechanism of proteins or protein texturization are still necessary. A great deal of information is essential to the process of optimization and attainment of the desirable structural and textural characteristics in the final freeze texturized protein product.

1.

Comparison of results from this work should be made with poultry meat residue proteins extracted by other means, for example, by salt extraction process. The data collected would provide further interpretation on the effect of alkali treatment of the proteins in relation to texturization.

2.

An investigation on protein polymerization should employ purified protein slurry in order to determine possible interferences by other solutes in the freezing system. This would determine the freeze-concentration-solubilizing effect (due to high salt content, presumably), which is thought to provide the cohesion and adhesion needed in the formation of the flaky, sheet structure in the texturized protein.

3.

Texturization of chemically modified proteins should be undertaken in order to determine the roles of some chemical bonds on intermolecular interactions during freeze concentration. One approach could be by succinylation of the amino acid residues (e.g. lysine), which would not only prevent peptide bond formation but also increase the negatively-charged groups along the polypeptide chains. Another approach is to work on the sulfhydryl-disulfide interchange reactions, by using reducing or oxidizing agents.

4.

X-ray analysis could be used to confirm the role of the various chemical bonds in determining protein structure and aggregation. In using this technique, it would be possible to deduce the sites of individual amino acids and the proximities of their functional groups, and the nature of the bonding forces involved in maintaining the folding and association of polypeptide chains can be established.

5.

It is known that highly cohesive films and other structures are attained by high molecular weight molecules that allow extensive molecular interactions. In the freeze texturization of poultry meat proteins the presence of high molecular weight protein subunits was indicated. Since the texture generated in this process is mainly due to compression by the ice crystals, the molecular size may not seem critical. However, it would be of great interest to texturize protein of various molecular sizes and shapes, so that these properties could be established for the protein material intended for texturization. Since the degree of molecular interaction could be measured in terms of tensile strength, this data would be helpful in formulating protein mixtures to meet the desirable textural characteristics required in the final textured product. The following food proteins are recommended on the basis of their molecular weights and shapes: meat residue proteins (fibrillar, high molecular weight), soy protein (globular, high molecular weight) and whey proteins (globular, low molecular weight), to be studied separately and in combination. This approach will establish not only the effects of molecular sizes and shapes of proteins but also the presence of covalent cross-links (disulfide bonds) in attaining extensive molecular interactions during freeze texturization.

5.3 Concluding Remarks

Freeze texturization is another method used to produce meat-like textured protein products. The process involves simple protein preparation steps and, in general, is considered as relatively economical compared to the other protein texturization techniques. Depending on the type of protein and processing conditions, production of the highly-defined fibrous structures is highly reproducible. Some of the major protein raw materials being considered for this process are the waste residual edible proteins from the food industry. There is still an enormous scope of the protein-type related problems associated with the freeze texturization process. However, any success in this area would bring about a considerable impact in the food industry (e.g. meat, dairy), especially in view of the magnitude of the current protein wastage.

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7. APPENDIX

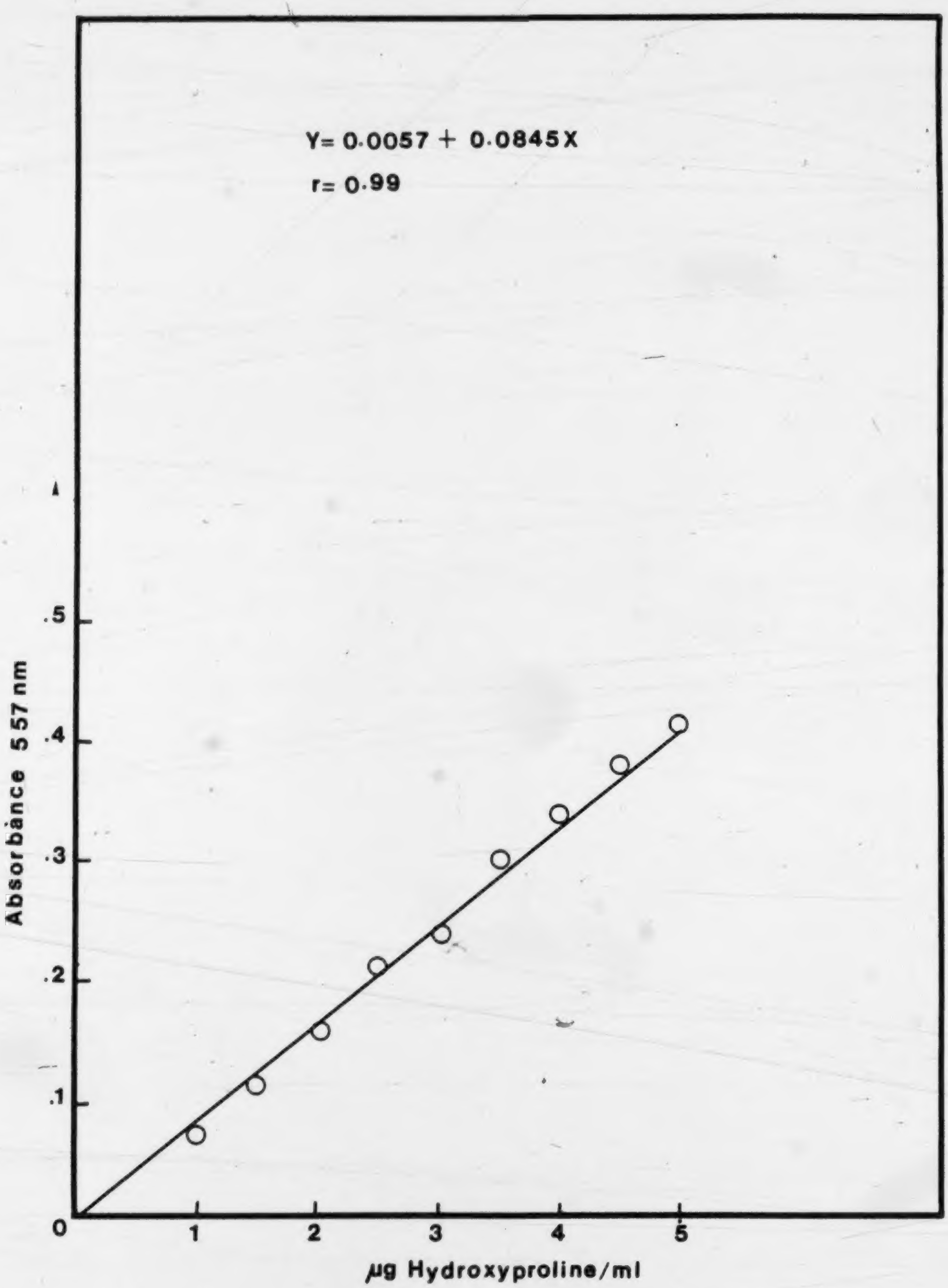

Figure 7.1 Hydroxyproline standard curve.

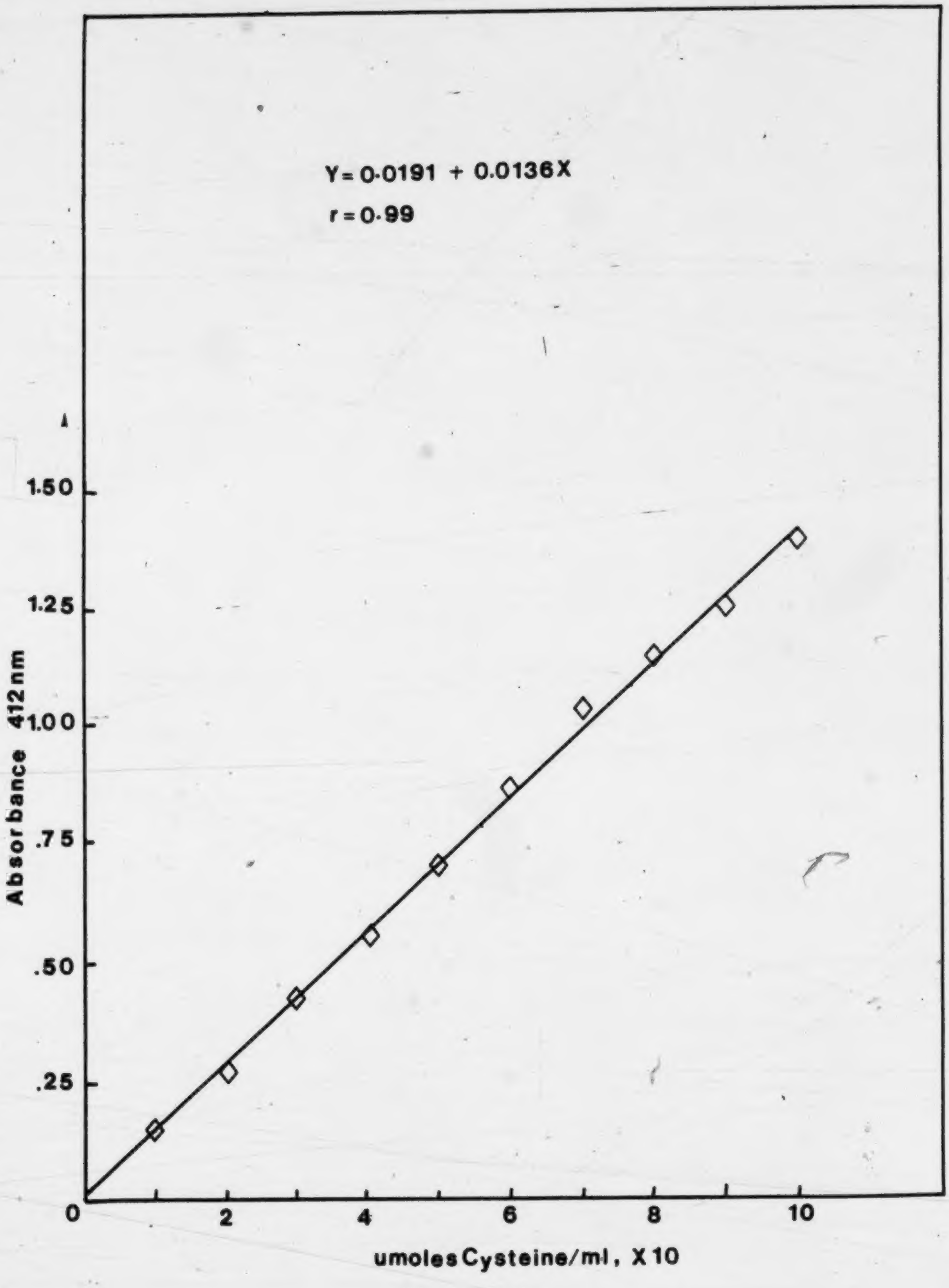

Figure 7.2 Cysteine standard curve.

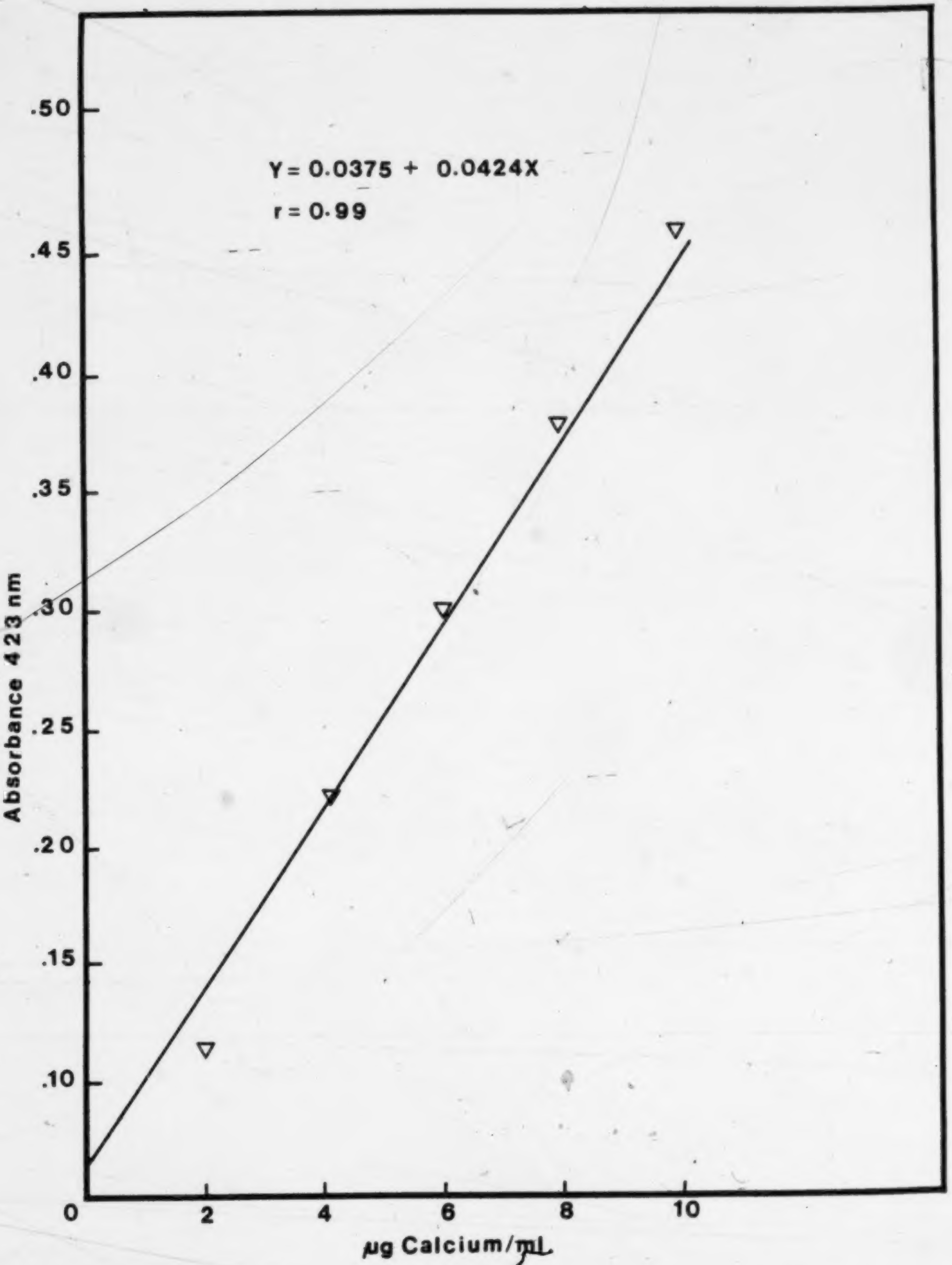

Figure 7.3 Calcium standard curve.

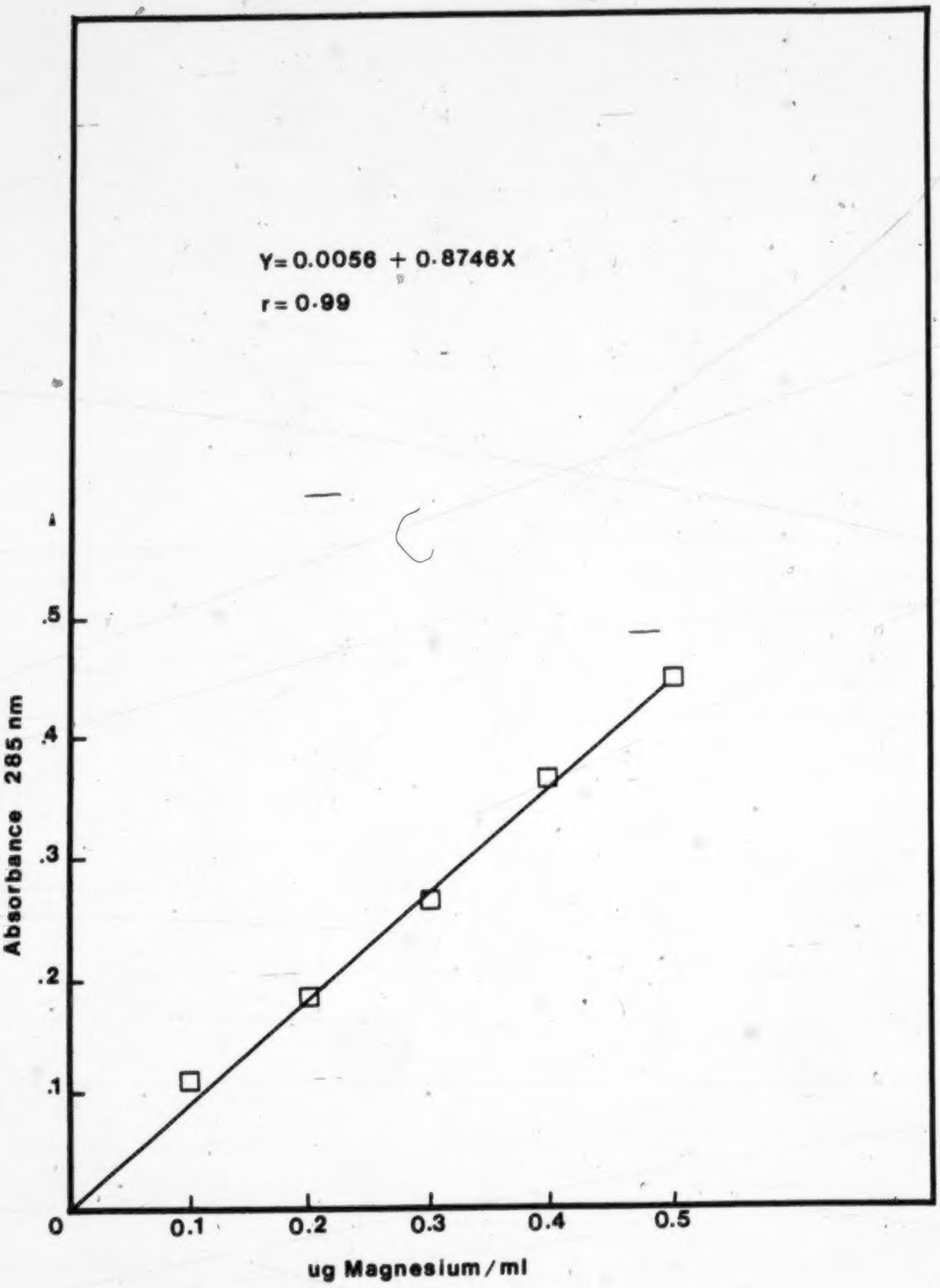

Figure 7.4 Magnesium standard curve.

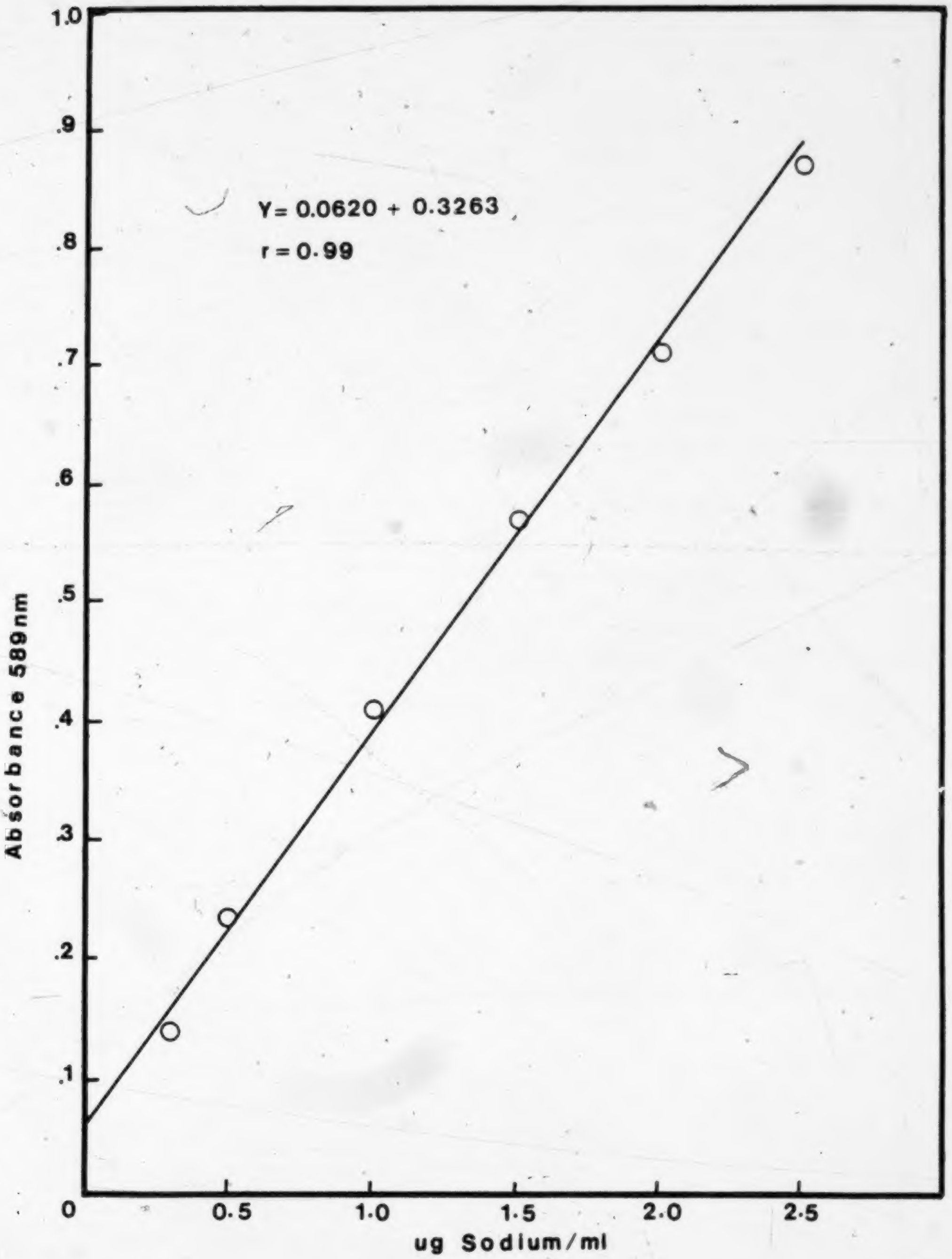

Figure 7.5 Sodium standard curve.

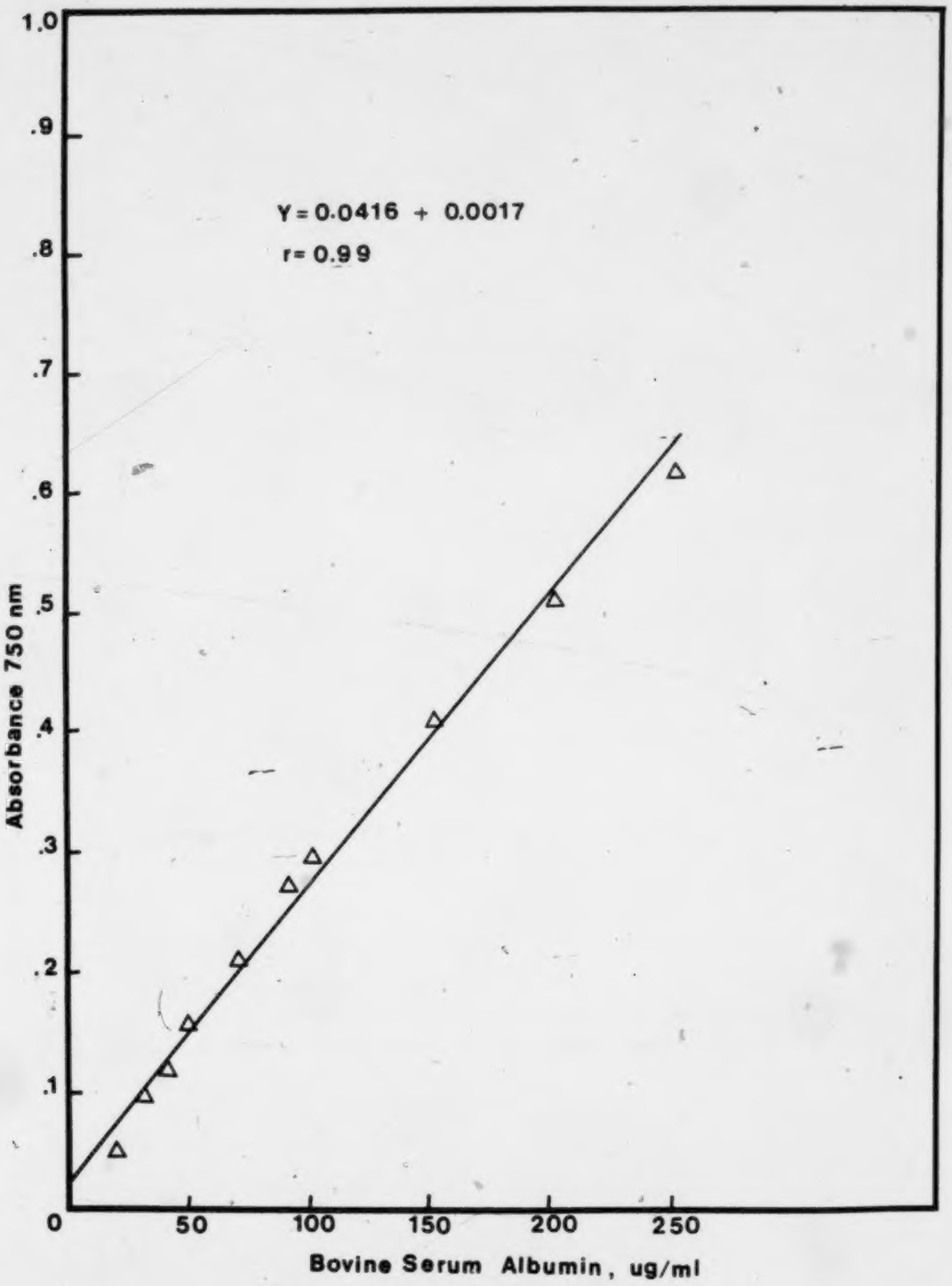

Figure 7.6 Bovine serum albumin standard curve.